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$$R^1\text{-}X^1\text{-[linker]-NHOH} \quad \text{(Ia)}$$
$$\quad\quad R^2$$

(57) Abstract

The invention provides a hydroxamate or hydroxamic acid compound of general formula (Ia) or (Ib): HONH–[linker]–$X^1R^1R^2$ or (Ic): $R^1R^2X^1$–[linker]–$X^1R^1R^2$, in which X^1 is a polar group selected from the group consisting of –C=O; $-COR^1$; $-CF_2$; $-CNH_2$; $-CNR^1$; $-SO_2-$; –P(O)(OH)–; –C=S; $-CSR^1$; –C–COR^1; –C–$CONR^1R^2$ and –C–CH_2OH; R^1 and R^2 are the same or different, and each is independently selected from the group consisting of H; OH; NH_2; NHOH; substituted or unsubstituted, branched or unbranched alkyl, alkenyl, alkylamino, alkyloxy or arylalkyloxy; substituted or unsubstituted aryl, aryloxy or pyridino; substituted or unsubstituted arylamino, piperidino, cycloalkyl, cycloalkylamino, pyridineamino, 9–purine–6–amine, and thiazoleamino; or either R^1 or R^2 is absent; and the linker is a group having a backbone of 5 to 9 atoms, which may comprise 1, 2 or 3 amino acids, or a pharmaceutically acceptable salt, ester or derivative thereof. The compounds have the ability to selectively prevent the growth of a variety of human tumour cell types, without affecting growth of normal cells. The compounds of the invention also inhibit the growth of protozoan parasites.

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HYDROXAMIC ACID COMPOUNDS HAVING ANTICANCER AND ANTI-PARASITIC PROPERTIES

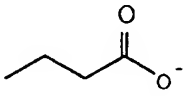
This invention relates to compounds which have anti-tumour and anti-parasite activity. The compounds are selectively cytotoxic against tumour cells without killing normal cell types, and can be used either alone or in combination with other anti-cancer agents. In particular, the invention relates to nitrogen-containing compounds, structurally related compounds, and derivatives thereof, which are selectively cytotoxic against tumour cells. The compounds of the invention characteristically inhibit deacetylation of histones and modify gene expression. The invention also provides pharmaceutical and/or veterinary compositions, and methods of treatment of cancer, of hyperplastic or dysplastic conditions such as psoriasis, leukoplakia, and solar keratosis, and of parasite infections, utilising the compounds of the invention. In addition, the invention provides methods for identifying particularly active compounds and for identifying patients likely to benefit from treatment with compounds of the invention.

BACKGROUND OF THE INVENTION

Cancer is one of the major causes of morbidity and mortality in modern society. Chemotherapy against cancer traditionally involves the use of cytotoxic agents such as anti-metabolites or DNA-targeting drugs that indiscriminately kill normal cells as well as tumour cells. These agents therefore cause serious side effects that are usually dose-limiting. Most such drugs are also ineffective or poorly effective against solid tumours. Thus new antitumour agents based on alternative mechanisms of action are needed to overcome these problems.

Cancer results from a series of genetic changes which usurp normal cellular mechanisms that control growth and morphology. Genetic mutation or loss has been associated with cellular transformation and cancer (Rueben

et al, 1976). One alternative method which may enable more selective targeting of tumour cells is conversion of cancerous cells to a non-proliferating phenotype through changes in gene expression. Studies of such reversion of oncogenically transformed cells to morphologically non-proliferating cells can provide valuable clues to aspects of the cell cycle which are still not fully understood. A number of compounds known to differentiate tumour cells, *eg.* butyrate (**1**), retinoic acid (**2**), and N,N'-hexamethylene-bis-acetamide (HMBA; **3**) (Marks *et al*, 1989) have undergone clinical trials, but all have suffered from problems of low potency, lack of selectivity, reversible differentiation or resistance.



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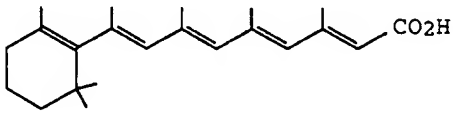

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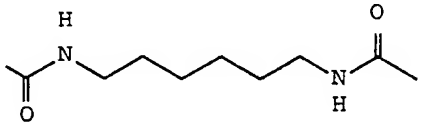

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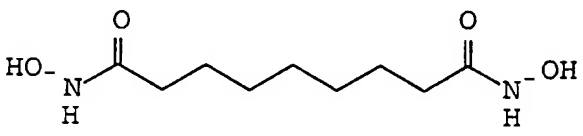
For example, HMBA has been reported to induce differentiation *in vitro* in many types of neoplastic (Young *et al*, 1988) and epithelial cell lines (Andreef *et al*, 1988) and embryonic cells (Egorin *et al* 1987). It has induced remissions in myelodysplastic syndrome and acute myeloblastic leukemia (Breslow *et al*, 1991), but suffers *in*

vivo from rapid degradation through deacetylation, and causes side effects such as thrombocytopaenia, neurotoxicity and acidosis (Marks *et al*, 1994).

In murine erythroleukaemic cells, HMBA induces arrest in G1, promotes translocation of protein kinase C (PKC) from the cytosol to the membrane, decreases c-myb, c-myc and p53 protein levels, and increases *c-fos* mRNA. A transient increase in hypophosphorylated retinoblastoma protein pRB was found 12 hr after treatment, followed by enhanced production of the hyperphosphorylated form ppRB during the next 2-3 days (Richon *et al*, 1992; Kiyokawa *et al*, 1994). A recently reported group of structurally-related but more potent differentiating agents induces differentiation in murine erythroleukaemic cell lines, human promyelocytic cells (HL-60), and human colon carcinoma cells (Breslow *et al*, 1991).

These workers have reported a family of compounds able to induce terminal differentiation (International Patent Applications No. PCT/US92/08454, published as No. WO 93/07148; No. PCT/US95/06554, published as WO 95/31977). The compounds, some of which are hydroxamates, are of general formula $R_1.CO.(CH_2)_n\ CO.R_2$, in which n is 4 to 8, and R_1 and R_2 may be the same or different, and are represented by a wide variety of substituents.

Azelaic bishydroxamic acid (ABHA; **4**) and some of its analogues were up to 100-fold more active than HMBA.



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A preferred compound is ABHA (**4**). The compounds are stated to induce terminal differentiation of neoplastic cells and thereby to inhibit proliferation of these cells,

but the activity requires prolonged contact of the compounds with the cells, preferably for at least 4 to 5 days. Activity against murine erythroleukaemia cells and acute promyelocytic leukaemia cells was observed in the range 1 to 2,500 μM (80 compounds tested, with one being inactive) and the range 1 to 20 μM (16 compounds tested) respectively. There is no disclosure or suggestion that any of the compounds, including ABHA, exerts selective toxicity against tumour cells, as distinct from normal cells. Nor is there any suggestion in the prior art that these compounds might have any activity against parasites.

We have now found that some of these compounds induce hyperacetylation of histone H4 when mammalian cells are treated in culture. Subsequent to the priority date of this application, this was independently confirmed (Richon *et al*, 1998). The histone deacetylase inhibitor trichostatin (TSA) has been proposed as a tumour-selective agent (Beppu *et al*, U.S. Patent No. 4,690,918, filed January 24, 1986), on the basis of modest *in vitro* selectivity against the short-term proliferation of SV40-transformed mouse fibroblasts compared with untransformed fibroblasts (see Table 3 in Beppu *et al*). This is not a satisfactory proof of concept for treatment of human cancer, because

(a) human cells were not used,

(b) SV40-transformed cells are not tumour cells and generally do not form tumours in animals,

(c) SV40 transformation of human fibroblasts can frequently produce sensitivity to alkylating agents which have no clinical value because of general lack of selectivity for tumour cells, and

(d) the 3-day observation period with continuous drug exposure was insufficient to show that the proliferation of the SV40-transformed cells was irreversibly inhibited.

Furthermore, we have shown in this application that TSA, although selectively toxic to some human tumour cells

in culture, is metabolised by cultured cells, and is inactive against human tumour cells *in vivo*. We have also shown that HC-toxin, a cyclic peptide proposed for the treatment of malaria (Darkin-Rattray *et al*, 1996), has properties similar to TSA, *ie*. it is tumour-selective *in vitro*, but is inactivated by cultured cells and lacks antitumour activity *in vivo*, even when used at high doses.

Melanocytes and melanoma cells express a range of differentiation markers related to pigment synthesis, including tyrosinase, HMB-45, melanin and the tyrosinase-related protein-1 (TRP-1) (Takahashi and Parsons, 1990; Sturm *et al*, 1994). During a comparison of the effects of azelaic acid, HMBA and the nine-carbon derivative ABHA on these markers we have now found, in contrast to the work of Breslow *et al* (1991), that in fact ABHA has poor activity as a differentiating agent with respect to pigmentation, which is the major differentiation pathway in melanocytic cells, and indeed that ABHA in some respects may act as a de-differentiating agent. We have also surprisingly found that ABHA was unusual, in being cytotoxic for transformed cells but not for normal cells, while activating the transcription of certain genes involved in signal transduction.

We have now identified a family of compounds which are selectively toxic against cell lines derived from over seven different human solid tumours, including a drug-resistant melanoma cell line and a lymphoid neoplasm. Typically these compounds do not have inhibitory activity against matrix metalloproteases. Particularly preferred compounds of the invention have been shown to be active *in vivo* against xenografts of the drug-resistant melanoma cell line in nude mice. It is particularly striking that the compounds of the invention are active against melanoma, ovarian cancer, and a range of other neoplasms which respond poorly to currently-available therapies. We have also demonstrated that compounds of the invention are

active against parasitic organisms, including drug-resistant strains of *Giardia* and *Plasmodium*.

SUMMARY OF THE INVENTION

This invention relates to compounds having the ability to selectively prevent the growth of a variety of human tumour cell types, without affecting the growth of normal human cells. In particular, the invention relates to nitrogen-containing compounds, structurally-related compounds, and derivatives thereof. In one preferred embodiment, the compounds are hydroxamate or bishydroxamate compounds or derivatives thereof. In another preferred embodiment the compounds are cyclic peptides. The compounds of the invention are selectively cytotoxic to human tumour cells both in cell culture, and in animal models *in vivo*. For the purposes of this specification, the expression "selectively cytotoxic to tumour cells" is to be understood to mean that proliferation of tumour cells *in vitro* is irreversibly inhibited, but normal cells are still able to proliferate, when exposed to a compound of the invention under comparable conditions.

In a first aspect, the invention provides a method of treatment of cancer, comprising the step of administration of an effective amount of a compound of the invention to a mammal in need of such treatment, said compound having selective cytotoxicity for neoplastic cells compared to normal cells, and having minimal or absent ability to induce differentiation in neoplastic cells.

Preferably the compound is selected from the group consisting of hydroxamates, bishydroxamates, or derivatives thereof, and cyclic peptides.

Typically the compound has the ability to inhibit deacetylation of histones.

Preferably the compound also enhances the *SphI*-containing promoter and/or the zinc-induced activity of the metallothionein Ia promoter.

Preferably the compound is selectively toxic to cells that express low levels of full length RbAp48.

More preferably the compound is not ABHA.

Typically, compounds of the invention either have no effect on, or down-regulate, expression of classical markers for differentiation in human melanoma cells. For example, TRP1 is down-regulated, while HMB-45and tyrosinase are not significantly affected.

It will be clearly understood that the method of the invention may be used in conjunction with one or more other anti-cancer therapies, such as chemotherapy or radiotherapy. In particular, it is contemplated that the method of the invention may be used in conjunction with one or more antiproliferative agents, such as cytosine arabinoside, 5-fluorouracil, , methotrexate, chlorodeoxyadenosine, etoposide, taxol (paclitaxel), and the like. The other treatment may be administered either concurrently, prophylactically or following the compound of the invention.

While it is contemplated that the compounds of the invention are useful for treatment of all types of cancer, including leukaemias and lymphomas, it is considered that these compounds will be particularly advantageous in the treatment of solid tumours, such as melanoma and other skin cancers, ovarian cancer, cervical cancer, breast cancer, prostate cancer, endometrial cancer, lung cancer, gastric cancer, colon cancer and the like.

Similarly, while the method of the invention is particularly contemplated for treatment of human cancer, it is also applicable to veterinary treatment. Thus the mammal may be a human, or may be a domestic, companion or zoo mammal, including but not limited to cattle, horses, sheep, goats, deer, cats, dogs, and large felids.

Preferably the compound is selectively toxic for at least one type of tumour cell, *ie.* the compound exerts a toxic or antiproliferative effect against the tumour cell but not against normal cells.

All the compounds of the invention have one or more of the following activities:

a) inhibition of growth in cell culture of at least one of the following human tumour cell lines: melanoma MM418cl, cervical HeLa, melanoma A2058, ovarian JAM, and lymphoma Mutu;

b) inhibition of growth in cell culture of transformed keratinocytes (HaCat) and melanocytes (Mel-SV);

c) inhibition of growth *in vivo* of human tumour cells (*eg.* melanoma MM96L) in xenografted nude mice;

d) inhibition of histone deacetylase, as measured by extent of hyperacetylation of histones;

e) induction of differences in protein expression by human tumour cells compared to normal human cells;

f) selective killing of tumour cells in (a) without killing normal cells;

g) blocking of cell cycle progression of some sensitive tumour cells in the G1/S phase;

h) induction of apoptosis in tumour cells; and

i) inhibition of DNA synthesis in normal but not in tumour cells.

Typically the compounds do not:

a) kill normal human cells, *eg.* melanocytes, neonatal foreskin fibroblasts (NFF), or peripheral blood lymphocytes, at dosages effective for killing human tumour cells;

b) significantly inhibit metalloproteinases known to be important for tissue maintenance and protein regulation, at a K_i equal to or less than 1 μM;

c) show overt signs of toxicity in animal models;

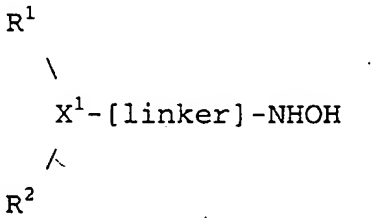
d) block the cell cycle of some drug-sensitive tumour cells at G_2/M phase.

The compounds of the invention, including ABHA, are effective in killing transformed keratinocytes.

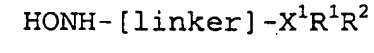
Therefore in a second aspect, the invention provides a method of treatment of a hyperplastic or dysplastic condition, comprising the step of administering an effective amount of a compound as defined above or of ABHA to a subject in need of such treatment.

Preferably the condition is a keratinous hyperplasia, such as psoriasis, leukoplakia or solar keratosis.

In a third aspect, the invention provides a hydroxamate or hydroxamic acid compound of general formula Ia or Ib or Ic,



Ia



Ib

Ic

in which X^1 is a polar group selected from the group consisting of -C=O; $-COR^1$; $-CF_2$; $-CNH_2$; $-CNR^1$; $-SO_2-$; -P(O)(OH)-; -C=S; $-CSR^1$; $-C-COR^1$; $-C-CONR^1R^2$ and $-C-CH_2OH$; or either R^1 or R^2 is absent;

R^1 and R^2 are the same or different, and each is independently selected from the group consisting of H; OH; NH_2; NHOH; substituted or unsubstituted, branched or unbranched alkyl, alkenyl, alkylamino, alkyloxy or arylalkyloxy; substituted or unsubstituted aryl, aryloxy or

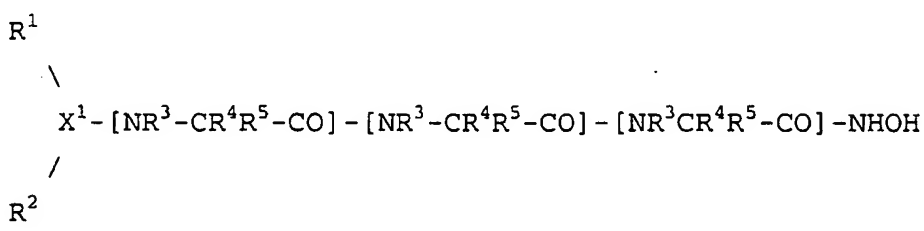
pyridino; substituted or unsubstituted arylamino, piperidino, cycloalkyl, cycloalkylamino, pyridineamino, 9-purine-6-amine, and thiazoleamino; and

the linker is a group having a backbone of 5 to 9 atoms, which may comprise 1, 2 or 3 amino acids,

or a pharmaceutically-acceptable salt, ester or derivative thereof.

In Formula Ic the two X^1 groups are independently selected from the listed groups.

Thus in one embodiment the compound is of formula II:



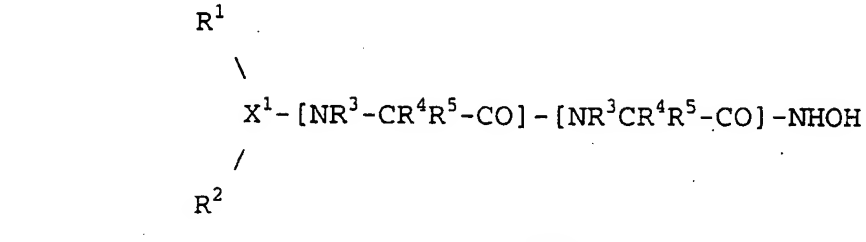
II

in which R^3 is as defined above for R^1 and R^2, and R^4 and R^5 are the same or different, and is each independently selected from H, alkyl, aryl or a side-chain of a common or uncommon amino acid.

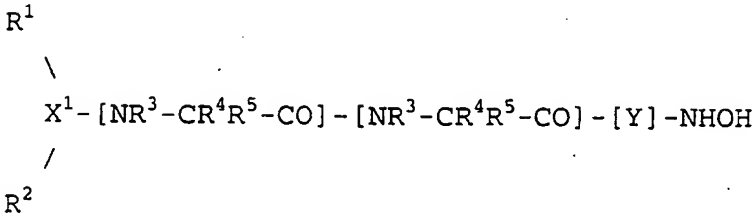
For the purposes of this specification, a "common" amino acid is a L-amino acid selected from the group consisting of glycine, leucine, isoleucine, valine, alanine, phenylalanine, tyrosine, tryptophan, aspartate, asparagine, glutamate, glutamine, cysteine, methionine, arginine, lysine, proline, serine, threonine and histidine.

An "uncommon" amino acid includes, but is not restricted to, D-amino acids, homo-amino acids, N-alkyl amino acids, dehydroamino acids, aromatic amino acids (other than phenylalanine, tyrosine and tryptophan), ortho-, meta- or para-aminobenzoic acid, ornithine, citrulline, norleucine, γ-glutamic acid, aminobutyric acid and α,α-disubstituted amino acids.

In another embodiment, the linker comprises two amino acids, and the compound is of formula IIIa or IIIb:



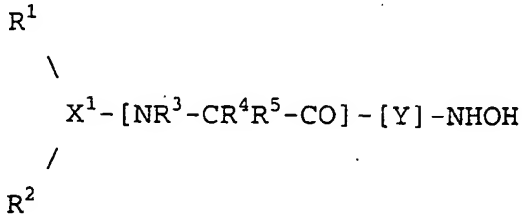
IIIa



IIIb

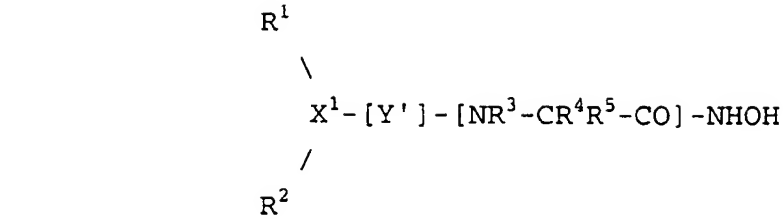
in which R^3, R^4 and R^5 are as defined above, and Y is $-CH=CH_2-CO$; $-C(alkyl)=C(H \text{ or } alkyl)_2$; $-C_6H_4-CO$; $-CH(alkyl)-CH(alkyl)-CO$; $-NR^6CH_2CH_2CO$; $-NR^6C(alkyl)-C(H \text{ or } alkyl)-CO-$; or $-NR^6-CH_6H_4-CO$, where R^6 is as defined above for R^1 and R^2, and alkyl is a linear or branched chain aliphatic group.

In a third embodiment, the linker comprises one amino acid, and the compound is of general formula IVa,



IVa

in which Y is as defined above, or formula IVb,

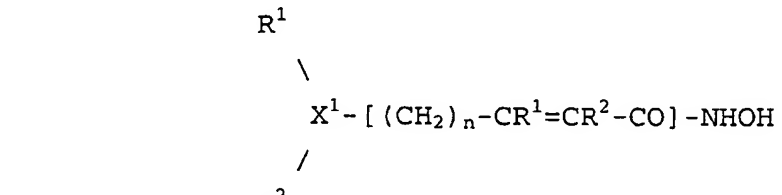


IVb

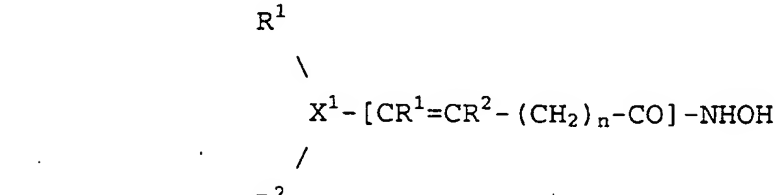
in which Y' is $-CH=CH_2-CO$; $-(CH_2)_n$, where n is an integer from 1 to 6; $-(CH_2)_3$; $-(CH_2)_4$; $-(CH_2)_2CO-$; $-(CH_2)_3-CO$; C_6H_4; $C_6H_4-CH=CH_2$; $-CH=CH_2-C_6H_4$; -CH(alkyl)-CH(alkyl); $-C_6H_4-CO$; $-C_6H_4-CH=CH-CO$; $-CH=CH-C_6H_4-CO$; or -CH(alkyl)-CH(alkyl)CO.

In both formulae IVa and IVb, R^3, R^4 and R^5 are as defined above.

In another embodiment, the linker comprises 1, 2 or 3 double bonds, and the compound is of the formula Va, Vb, Vc, Vd Ve, Vf or Vg,



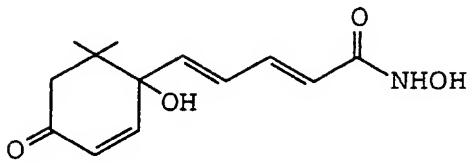
Va



Vb

Vc

Vd



Ve

Vf

Vg

in which R^1 and R^2 may be the same or different, and are as defined above;

where each of n and m is independently an integer from 1 to 6,

and in which the C_6H_4 group is an aromatic ring, optionally substituted at the ortho-, meta- or para-position with a substituent selected from the group consisting of NO_2, NH_2, NMe_2, Cl, F, SO_2NH_2, Me and alkyl.

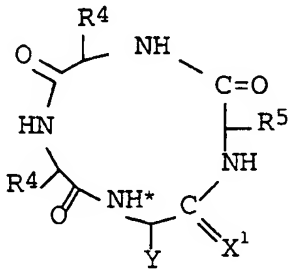
Preferably each of R^1 and R^2 is independently H, alkyl or aryl,

In an alternative embodiment, the polar group X^1 forms part of a cyclic tetrapeptide of formula VI,

$$cyclo[(CX^1\text{-}CHY\text{-}NH)(COCHR^4NH)_3]$$

VI

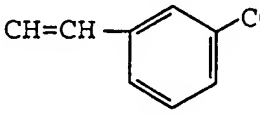
for example



VII

in which each of R^3, R^4 and R^5 are the same or different, and are as defined above, or may be thioproline, hydroxyproline, pipecolic acid, or decahydroisoquinoline;

X^1 and Y are as defined above, or Y may be $(CH_2)_5COMe$, $(CH_2)_4COMe$, $(CH_2)_5CO$-alkyl, $(CH_2)_5CO$-aryl, or $(CH_2)_5CO\text{-}NR^3R^6$, wherein R^3 and R^6 are the same or different, and are as defined above.

Preferably each of R^3 and R^6 is selected from the group consisting of H, alkyl, aryl, $(CH_2)_5CHO$,  ; and CH=CH-CH=CH-CH_2-COMe; and one or more of the four amino acids is optionally N-alkylated with an aliphatic alkyl group.

The stereochemical configuration at the position marked by * may be R or S (L or D).

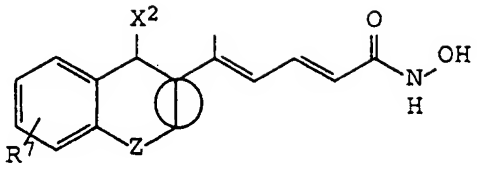
In another alternative embodiment, the polar group X^1 forms part of a cyclic pentapeptide, and the compound is of formula VIII,

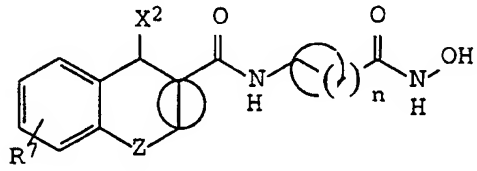
cyclo[CX^1-CHY-NH)($COCHR^4NH)_4$]

VIII

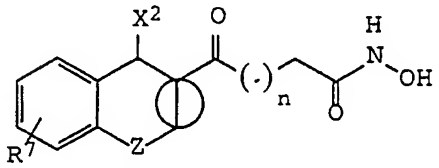
in which X^1 and Y are as defined for the previous embodiment, and the other substituents are as defined above.

In yet a further embodiment compounds of the invention are cyclic molecules, such as quinolines, isoquinolines, tetrahydroquinolines, or decahydroquinolines; or a compound selected from the group consisting of

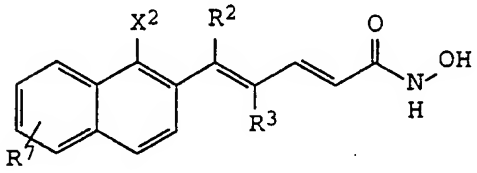


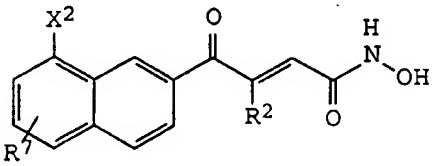


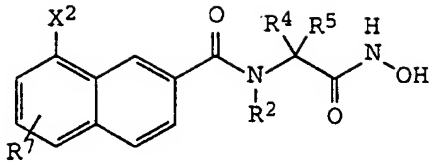
n=0,1,2

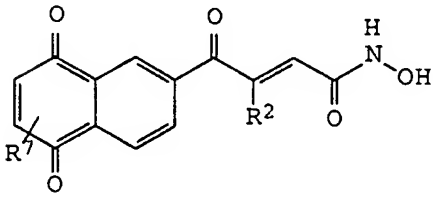


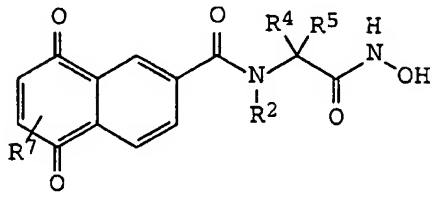
n=1,2,3

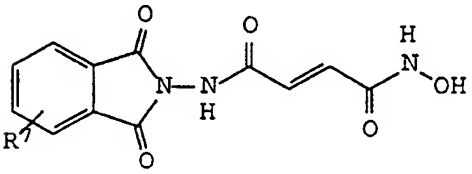


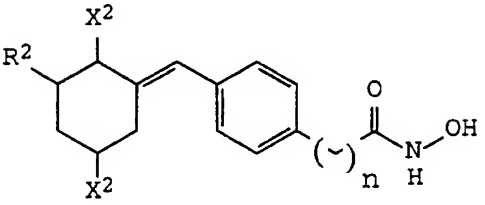




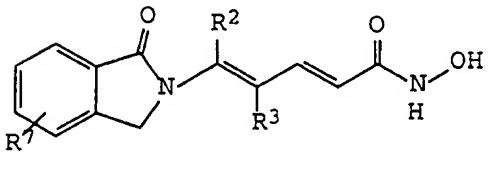


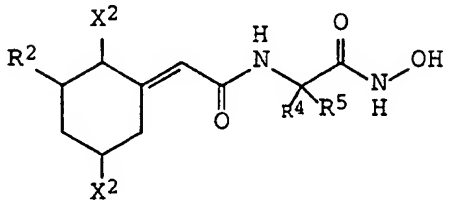


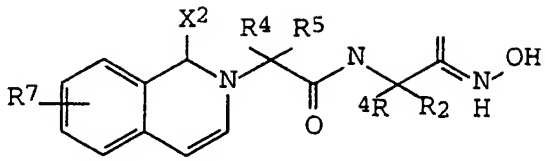


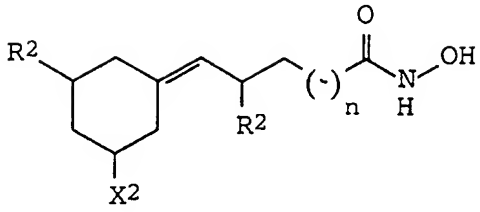


n=1.2



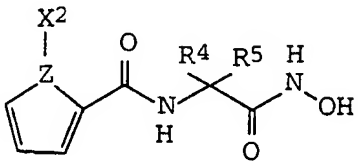






n=0,1,2

and



in which Z is O, S, NH, N-alkyl; NO; SO; CO; C-R^7;

X^2 is O, OH, aldehyde, ketone, CF_3; NO_2; NO; SH; S; NH; NH_2; CO_2H; $CONH_2$; CO_2(alkyl); CONH(alkyl); or other polar group;

R^7 is one or more substituents such as H; OH; OMe; NO_2; Cl; Br; F; $(Me)_2N$; CN; NH_2: NH(alkyl); $N(alkyl)_2$; SO_3H; SO_2NH_2; alkyl CF_3; O(alkyl); SH; S(alkyl) *etc*, and in which

each bond depicted as an alkene bond may alternatively be a single bond, and each single bond marked with a circle may alternatively be a double bond.

In a fourth aspect the invention provides a composition comprising a compound of the invention, general formulae or a pharmacologically acceptable salt, ester or derivative thereof, together with a pharmaceutically or veterinarily acceptable carrier.

The specifications of WO 93/07148 and WO 95/31977 describe the ability of the compounds disclosed therein to induce terminal differentiation and to inhibit proliferation of neoplastic cells; there is no disclosure or suggestion of activity in hyperplastic or dysplastic conditions, modulation of immunological activity, or active against protozoal parasites. Consequently, it will be clearly understood that the following aspects of the

invention encompasses the use of compounds disclosed in WO 93/07148 and WO 95/31977 in addition to the novel compounds of the invention. These compounds are referred to herein as "ABHA and related compounds".

We have also surprisingly found that compounds of the invention, and ABHA, at a dose of 0.1 to 150 µg/ml inhibit the growth of protozoan parasites such as *Giardia duodenalis* and *Plasmodium falciparum in vitro* or *in vivo*.

Therefore in a fifth aspect, the invention provides a method of treatment of a protozoal parasite infection, comprising the step of administering an effective dose of one or more compounds of the invention, or of ABHA or a related compound, to a subject in need of such treatment.

It is contemplated that this aspect of the invention is applicable to the treatment of infection with a variety of protozoal parasites, including but not limited to protozoa of the genera *Giardia*, *Cryptosporidium*, *Trichomonas*, *Histomonas*, *Plasmodium*, *Toxoplasma*, *Trypanosoma*, *Babesia*, *Balantidium*, *Naegleria*, *Entamoeba*, *Eimeria*, *Schistomaniasis*, other intestinal parasites, and the like. Many of these parasites present major public health or veterinary problems, particularly in developing countries, and currently-available therapies are unsatisfactory.

Preferably the parasite is *Giardia*, particularly *Giardia duodenalis*, *Plasmodium*, particularly *Plasmodium falciparum*, or *Trichomonas*, preferably *Trichomonas vaginalis*.

Preferably the compound is AAHA, MW2796, MW2996, as herein defined, or ABHA. More preferably the compound is MW2796.

We have shown that selective cytotoxicity of the compounds of the invention is facilitated by low or aberrant expression of the retinoblastoma binding protein RbAp48.

Therefore in a sixth aspect, the invention provides a method of identification of cancers which are particularly amenable to treatment by the method of the invention, comprising the step of detecting abnormal levels or absence of full length RbAp48 in a sample of the cancer. Suitably this may be achieved by subjecting a histological section of the tumour, obtained via biopsy or at the time of surgical excision of the tumour, to immunohistochemical analysis with an antibody directed to RbAp48. The antibody may be labelled with any suitable detectable marker, and fluorescent or radioactive markers are preferred. Fluorescent markers are particularly preferred. Suitable methods will be well known by persons skilled in the art.

The compounds of the invention may be administered by any suitable route, including but not limited to parenteral injection, for example intravenous, subcutaneous, intramuscular and intratumoural injection, oral administration, transdermal and topical administration. In general oral, transdermal or topical administration is preferred. It is expected on the basis of the general chemical properties of hydroxamates and related compounds that at least some compounds useful for the purposes of the invention will be orally bioavailable, and we have demonstrated that this is the case. Structural modifications whereby oral bioavailability can be improved are known in the art; see for example Beckett *et al*, 1996.

The dose and route of administration will depend on the nature of the cancer or protozoal parasite infection to be treated, any other treatments which have been administered or which are also to be used, and the general state of health of the subject, and will be at the discretion of the attending physician or veterinarian. It is contemplated that a suitable dose range will be 0.1 to 100 mg/kg body weight, administered in single or divided doses. For example, three separate doses per day may be used. Alternatively continuous infusion via a pump may be used. A wide variety of suitable carriers and formulation

agents is known in the art, and reference may be made for example to Remington's Pharmaceutical Sciences, 19th Edition, Mack Publishing Company, Easton, Pennsylvania.

The formulation will depend on the dose and route to be used, and is a matter of routine trial and error experimentation.

In a seventh aspect, the invention provides a method of enhancing the selectivity of treatment of a cancer or of a protozoal parasite infection with a compound of the invention, comprising the step of administering a nucleic acid sequence complementary to a nucleic acid sequence encoding RbAp48 or to an SphI-containing sequence to the subject to be treated. Preferably the complementary sequence is targeted to tumour cells or to protozoal parasites.

In an eighth aspect, the invention provides a method of increasing the proportion of tumour cells recognised by the immune system, comprising the step of administering a compound of the invention to a subject suffering from the tumour, thereby to increase the proportion of tumour cells expressing MHC Class I molecules. This relates to use of compounds of the invention, or of ABHA and related compounds, to modulate immune responses of patients with cancer or any other conditions where enhancement of the immune system or of immunoregulatory molecules is required.

It will be clearly understood that the invention insofar as it pertains to compounds *per se* and to pharmaceutical compositions *per se* for the treatment of cancer, does not include azelaic bishydroxamic acid or compounds disclosed in WO 95/31977 or WO 93/07148.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to" and the word "comprises" has a corresponding meaning.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows the enhanced dendritic morphology induced in melanoma cells (A,B) or HeLa cells (E,F) but not in normal melanocytes (C,D), or neonatal foreskin fibroblast cells (NFF, G,H). Inhibitor: azelaic bishydroxamic acid (ABHA), 100 µM.

Figure 1B shows apoptosis (programmed cell death) in cells treated with 100 µg/ml ABHA for 24 hr, fixed in methanol and stained with Hoechst 33248. Apoptotic cells have fragmented nuclei. Left panels, untreated cells. Right panels, treated cells. A,B: MM96L cells. C,D: HeLa. E,F: MM229 cells. G,H: human neonatal foreskin fibroblasts (NFF).

Figure 2 shows flow cytometry profiles illustrating the ability of compounds of the invention to block cell proliferation. Tumour cells (MM96L and HeLa) are sensitive, while normal cells (NFF, D29) are resistant. TSA: trichostatin A; Mk-4: same as azelaic-1-hydroxamate-9-anilide (AAHA) ; HU: hydroxyurea. Doses of ABHA and AAHA are in µg/mL; doses of TSA are in ng/mL; Numbers indicate dose; e.g.: ABHA 3 = 3 µg/mL ABHA; Horizontal axis: DNA content.

Figure 3 illustrates the selective inhibition of tumour cell growth by ABHA.

Panel A shows the response for melanocytes (●),neonatal foreskin fibroblasts (■), HaCat transformed keratinocytes (Δ), Mel-SV melanocytes (▲) and MM418c1 melanoma (O).

Panel B shows results for the tumour cell lines HeLa cervical (□), A2058 melanoma (O), MM96L melanoma (●) and JAM ovarian carcinoma (Δ). Each point represents the mean ± standard deviation (n=3).

Figure 4 shows the loss of hyperphosphorylated retinoblastoma protein (RB) in cells treated with ABHA (13 µg/mL) or hexamethylene bisacetamide (HMBA: 1,000 µg/mL) for either 12 hr or three days. ppRB represents

hyperphosphorylated RB (115 kDa); pRB represents hypophosphorylated RB (105 kDa).

Figure 5A shows the dose response of ABHA for modulation of sheep metallothionein Ia promoter in the presence of 100 μM $ZnSO4_4$ (□ MM96-Gal; O HeLa-Gal). Each point represents the mean ± standard deviation (n=3).

Figure 5B shows the induction of transcription in HeLa cells after 24 hr treatment with ABHA or AAHA, using a variety of promoter constructs driving the luciferase gene and measured as luciferase activity by luminescence. The *SphI* sequence is identified as the sequence essential for full activation. Oct: octamer sequence derived from the SV40 enhancer region, and inserted into the pGL2 reporter construct; SphI: the *SphI* sequence; SphII: the *SphII* sequence; wt : wild type; dpm2, dpm7 and dpm8: similar sequences containing the mutations identified in bold type

Figure 5C shows that the *SphI* activity is induced by all histone deacetylation inhibitors tested, but only in cells expressing the tumour-suppressor gene, p16.

Figures 6A, 6B and 6C show growth of xenografted MM96L human melanomas in nude mice which had been treated with ABHA at 4 mg/day, AAHA at 5 mg/day, SBHA at 8 mg/day, TSA at 25 μg/day or HC-toxin at 25 μg/day. ● = treated with ABHA, AAHA or SBHA; Δ = treated with TSA or HC-toxin; □ = untreated controls (n=7-11 per group).

Figure 7A shows the results of two-dimensional polyacrylamide gel electrophoresis comparing cell extracts of the melanoma cell line MM96L (a) with and (b) without treatment with 100 μg/ml ABHA. Small black arrows indicate proteins that are lost due to drug treatment; small white arrows indicate proteins that are gained due to drug treatment.

Figure 7B shows two one-dimensional PAGE gels of proteins separated from control and treated cells. Bold arrows show proteins that are specifically induced by ABHA (100 μg/ml for 24 hr) or by UBV (240 Joules/m^2 radiation for 24 hr) in sensitive cells. The cells used were MM96L

and A2058 melanoma cell lines, MEL/SV40 SV40-transformed melanocytes and NFF cells. * shows the location of BSA derived from the FCS, the presence of which obscures this region of the gel. M: molecular weight markers; C: control.

Figure 7C shows a western blot of MM96L cells, reacted with OV9D1 antibody to Ku86. The level of this protein (at 86 kDa) is upregulated in the cytosol (C) and depleted in the nucleus (N) by ABHA treatment (100 µg/ml for 24 hr).

Figure 8 shows a pharmacophore model in which the structures of active (grey) and inactive (white) compounds are overlaid.

Figure 9A shows acetylation of histone H4 during 24 hr treatment of MM96L cells with 100 µg/ml ABHA (lane C: control, lane A: ABHA treated) or treatment of HeLa cells with 5 mM sodium butyrate (lane C: control; lane B:, treated); H: histone mixture. Bands were visualised by protein staining of polyacrylamide gel separated acid-soluble material.

Figure 9B also shows acetylation of histone H4 during 24 hr treatment of MM96L, this time with 100 µg/ml ABHA, and of NFF cells with 100 µg/ml ABHA. Bands were visualised by protein staining of polyacrylamide gel separated acid-soluble material. '-' = without ABHA; '+' = with ABHA; H2A, H1, H3, H2B are other histones.

Figure 9C shows a laser densitometry trace of the time course of histone H4 acetylation during treatment with ABHA, in MM96L cells (top panels) and NFF (lower panels). H2A, H1, H3 and H2B are other histones; 4, 3, 2, 1 and 0 represent the number of acetyl groups attached; e.g. 4 = tetra-acetylhistone H4.

Figure 9D shows the rate of loss of acetylated histone H4 after removal of ABHA from the culture medium. Cells had been treated previously with 10 µg/ml ABHA for 24 hr.

Figure 10A shows the levels and relative sizes of proteins in cell lysates reactive with RbAp48 antibody, as revealed by western blotting. Sensitive cell lines (*eg.* MM96L, HeLa) had low levels of full length protein when compared with the resistant cell lines MM229, NFF, Melanocyte and A2058.

Figure 10B shows that hybrids between sensitive (HeLa) and resistant (NFF or A2058) cells tend to express a pattern of RbAp48 proteins that resembles that of the sensitive parent line; *ie.*: there is less full length protein (48kDa) than would have been expected from the parent resistant line. A/H mp2, A/H B5 are A2058/HeLa hybrids. N/H mp are NFF/HeLa hybrids.

Figures 11A and B show the instability of 100 ng/ml TSA (A) and HC-toxin (B) in the presence of cultured cells, compared with ABHA. The dose response for inactivation of HC-toxin (panel B) was conducted using 24, 48 and 72 hr exposures to MM96L cells and compared with incubation in culture medium alone for 72 hr. Drugs were incubated in microtitre plates with 50,000 MM96L cells per well, and at various times the medium was transferred to another plate containing 5000 MM96L cells for a 24 hr treatment, followed by a further 5 day incubation for determination of cell survival.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only to the following general methods and examples, and to the figures.

Abbreviations

AAHA	azelaic-1-hydroxamate-9-anilide (Mk-4)
ABHA	azelaic bishydroxamic acid
DMEM	Dulbecco's modified Eagle's medium
FCS	foetal calf serum
HMBA	hexamethylene bisacetamide
MSMS	mass spectroscopy mass spectroscopy

NFF	neonatal foreskin fibroblasts
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline, pH 7.3
pRB	retinoblastoma protein
PKC	phosphokinase C
PVDF	polyvinyl difluoride membrane
RbAp48	pRB binding protein
SBHA	suberic bishydroxamic acid
SphI	8bp nucleotide sequence derived from the Simian Virus 40 (SV40) enhancer region
TPA	12-O-tetradecanoylphorbol 13-acetate
TRP-1	tyrosinase-related protein-1
TSA	Trichostatin A.

Cell Culture

The origins of the human melanoma cell linesMM96E, MM96L (subclones of MM96), MM418c1 and MM418c5, the human cervical tumour cell line HeLa and the ovarian tumour cell line JAM (McEwan *et al*, 1988; Wong *et al*, 1994) and the spontaneously transformed keratinocyte line HaCat have been described previously. NFF were human neonatal foreskin fibroblasts. Normal human melanocytes from foreskins were cultured in 100 µg/mL, 12-O-tetradecanoyl-phorbol 13-acetate and 6 µg/ml cholera toxin. The mel-SV line of immortalised human melanocytes, obtained after infection of melanocytes with an SV40-adenovirus 5 hybrid virus, was kindly provided by Prof. P. Gallimore (Birmingham, U.K.) Cultures were grown at 37°C in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% foetal calf serum (FCS) as previously described.

Cell survival was determined by performing haemocytometer counts of the increase in number of 25,000 cells seeded in 24-well plates (16 mm diameter wells) and treated with drug for 24 hr, washed twice and incubated for 3 doubling times (6 days for NFF, melanocytes and MM418; 3 days for the other cell lines).

Alternatively cell numbers are determined with the MTS/PMS method or by assessing ^{3}H-thymidine incorporation, which gives similar results (Parsons *et al*, 1997). Assays for galactosidase action using chlorophenol red galactoside (CPRG) substrate may be performed in microtitre plates and read using a ELISA reader (Wong *et al*, 1994).

In the functional tests described below, treated cells were compared with controls on the basis of equal cell number or protein content, the latter determined by addition of bichinchoninic acid reagent (Pierce Chemical Co, USA) to triplicates in a microtitre plate and determining the absorbance increase at 570nm. Bovine serum albumin was used as the standard.

Synthesis of Azelaic bishydroxamic acid (ABHA)

To a solution of azelaic acid (nonanedioic acid; 1.0 g; 5.3 mM) in dimethylformamide (25 ml) was added triethylamine (2.68 g; 3.7 ml; 26.5 mM), hydroxylamine hydrochloride (0.77 g; 11 mM and benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (6.2 g; 14 mM) and the reaction mixture stirred under nitrogen at room temperature for 12 hr. The mixture was then diluted with water (100 ml) and lyophilised to yield the crude product as a thick syrup. This material was redissolved in a mixture of acetonitrile (4 ml) and water (16 ml), filtered, and chromatographed by HPLC (Waters deltapak C18,15 mm, 40 x 100 mm, flow 20 ml/min, eluant 20% acetonitrile/80% water/0.1% trifluoroacetic acid). The purified material was lyophilised to yield a white powder (0.92 g; 80%), which was identical by ^{1}H NMR spectroscopy and electrospray mass spectrometry with that previously reported (Breslow *et al*, 1991).

Tyrosinase Activity and Pigmentation Antigens

Tyrosinase (dopa oxidase) activity was measured by the oxidation of L-dopa as described (Takahashi and

Parsons, 1990). Immunoblotting was conducted using B8G3 mouse monoclonal antibody supernatant against TRP-1 or HMB-45 mouse antibody (diluted 1 in 250), followed by alkaline phosphatase-conjugated anti-mouse antibody, and was quantitated by a Molecular Dynamics laser densitometer with ImageQuant software.

Analysis of Transcriptional Regulation Using LuciferaseTransfectants

Clones of MM96L and HeLa cells stably transfected with reporter plasmids were used to determine the effect of ABHA on promoter activities. Construction of the TRP-1 promoter has been described (Sturm *et al*, 1994). The SV40 promoter/enhancer construct was obtained from Promega. For construction of a reporter containing a p53 response element, a duplex oligonucleotide containing a strong palindromic p53 recognition site (Funk *et al*, 1992) was cloned as a blunt-ended fragment

(5'CCGTCTGGACATGCCCGGGCATGTCCTCTCC)

into the blunt Ec1 136II site of the enhancer-probe vector pGL2-promoter (Promega). The structure of plasmids containing a single inserted oligonucleotide was confirmed by automated DNA sequencing (Taylor and Dunn, 1994), with primers allowing reading of both strands of the insert. Each of the above response elements was coupled to a luciferase reporter gene. Stably transfected cell clones were picked after co-transfection with a Neo resistance plasmid and selection with 400 µg/ml Geneticin. Cells were seeded at 50,000/well in black microtitre plates with clear, cell culture grade bottoms and incubated with drug for 6 or 24 hr. The medium was then replaced with 20 µl of Promega luciferase assay reagent and luminescence counting performed immediately *in situ* with a Packard Top Plate instrument.

Chemical Design and Synthesis

Compounds were synthesized using well-established organic chemistry solution and solid phase techniques. Combinatorial libraries were constructed using Combi-chem multipins, obtained from Chiron. Purification was effected by column chromatography on silica gel or by rp-HPLC using acetonitrile-water eluants. Compounds were characterised by mass spectrometry and NMR spectroscopy.

Matrix Metalloproteases

Compounds were screened in batches against a battery of metalloproteases, using conventional methods, for example assays for the matrix metalloproteins stromelysin (MMP-3 : EC3.4.24.17), human neutrophil collagenase (MMP-8: EC 3.4.24.34), and inhibitors of TNF-alpha convertase. Suitable assays are described in Birkedal-Hansen (1993) and in McGeehan *et al* (1994).

Histone Hyperacetylation

Histone hyperacetylation was determined by acid extraction of nuclear proteins, followed by PAGE or Triton/urea gels, and staining of gels with Coomassie blue stain. Alternatively, hyperacetylation may be determined indirectly by accessibility of DNA to Hoechst 33258, using flow cytometry, and directly by PAGE. Levels of histone deacetylase and acetyl transferase are measured by Western blotting, or by RT-PCR from the published sequences. cDNA is used for constructing expression vectors, as carried out for Brn-2 antisense sequences (Thompson *et al*, 1995).

Xenografts of Melanoma

Xenografts of melanoma MM96L, HeLa and the ovarian cancer cell line JAM were grown in nude mice (6 per group, 2 sites per mouse on the flanks) as described previously (Parsons *et al*, 1991). After 1 week to establish tumours, drug treatments in saline were given i.p. once daily to 3 mice at a concentration just below any

toxic level found, then at a lower, more efficacious dose to all 6 mice in each group. In initial experiments with BALB/c mice, no general toxicity has been found for ABHA. The size of tumours was measured weekly, and animals euthanised when the tumour reached 1 cm diameter. If any tumours developed in treated mice, tumour cells were isolated back into culture to determine if resistance had developed.

Receptor Identification

a) 2D PAGE

Cells were grown in the presence or absence of 10 µg/mL test compound in normal medium for 24 hr. Cells (10^6) were then harvested, washed with cold saline and lysed. Cell membranes were removed by centrifugation and protein extracts loaded on to immobilised pH gradient (IPG) gels for isoelectric focussing. Focussing normally requires 250,000 volt/hours. The IPG gels were then separated in polyacrylamide gels (4%-15%) and silver stained. In a modification, the protein extract is first mixed with 10 mg/mL of radiolabelled differentiating agent and viewed by autoradiography at the end of the experiment. Specific proteins can be extracted directly from acrylamide gels or electroblotted onto PVDF membrane. Using preparative 2D PAGE techniques, it is possible to load up to 50 mg of proteins in a complex mixture. Depending on the level of expression, this may require partial purification of protein extracts (prior to loading) by molecular weight partitioning with "centricons" or by size exclusion chromatography. Larger scale cell culture (up to 10^9 cells) may also be required. Protocols used in all studies are similar to those used in the Australian Proteome Analysis Facility (APAF), Macquarie University.

b) Biacore™ Analysis

A suitably functionalised differentiating agent is linked to the surface of a CM-5 chip through a series of

glycine residues using standard solid phase peptide coupling. Cell lysates are then fractioned to remove particulate matter and passed over the immobilised differentiating agent. The immobilised material is then washed with buffered saline and desorbed (usually by changing pH or ionic strength). Desorbed material can be collected and run on gels to identify which bands in the 2D-PAGE experiments are binding. Up to 30 ng of protein is collected from a single Biacore™ experiment, although this depends on the strength of the interaction and number of receptors. It may be necessary to prepare an affinity column to obtain sufficient protein for analysis.

c) Affinity chromatography

The compound is linked to the solid support Tresyl Sepharose or similar. Cell lysates (10^8 or more cells) prepared in 0.5% Triton X-100 are diluted into low salt buffer and after washing unbound material away Sepharose-bound proteins are eluted with batches of NaCl (to 2M) and finally by electroelution. Fractions are run on PAGE gels with silver detection of proteins. Relevant bands from a large scale preparation are transferred to PVDF membrane and the protein N-terminus sequenced by MSMS and tryptic digests.

Pharmacological Profiles

LogP values are predicted using PALLAS pKalc and PrologP programs (Compudrug Chemistry Ltd, Hungary), and measured using a HPLC elution rates and a standard calibration curve with control compounds of known LogP.

Cell Uptake Experiments

HeLa cells, human fibroblasts or any other desired test cells are seeded at 10^6 cells per mL and grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum (FCS) at 37°C. After 20 hr, medium is removed, cells are washed twice with 5 mL of

medium (without FCS) and test compound labelled with a suitable isotope is added to cells at varying concentrations (0.05 - 1 mM) in DMEM. Cells are grown for 20 hr more, washed twice with ice-cold DMEM, and counted in a liquid scintillation counter.

Example 1 Morphology and Cell Survival

Within 12 hr of commencing ABHA treatment, cells revealed characteristic morphological changes. The pigmented line, MM418c5, and the amelanotic melanoma line MM96E altered from spindle shape to markedly elongated cells with long processes. HeLa morphology changed from cuboidal to a more elongated, spindle-shape, with many cells possessing distinct processes. This is shown in Figure 1A .

In contrast, the transformed kidney cell line 293 formed large aggregates of cells instead of flattened colonies. Cells ceased to proliferate after several days treatment, but there were no signs of overt toxicity until higher doses were used (100 µg/ml ABHA). Apoptotic cells were detected, as shown in Figure 1B, and tended to be more numerous in sensitive cell types (MM96L, HeLa) than in resistant cells (NFF, MM229). This is summarized in Table 1.

Table 1

Apoptosis Induced in Human Cells by 24 hr Treatment with 100 µg/ml ABHA

Cell Line	% Cells Undergoing Apoptosis[a]	
	Control	ABHA-treated
MM96L	0	25
HeLa	0.5	5.0
MM229	0	3.0
NFF	0	2.5

[a] Determined by scoring 3 fields (30-158 cells/field) under the fluorescence microscope, after staining fixed cells with 5 µg/ml Hoechst 33248.

Melanoma cells were considerably more sensitive than HeLa cells, and a higher proportion of the former became arrested in G1. This is illustrated in Figure 2, which shows the effect of the hydroxamates ABHA and AAHA on the cycling of sensitive (MM96L, HeLa) and resistant (NFF, 229) cells. Hydroxyurea (HU) was included as a control agent to show that the cells could be blocked in G1, and Trichostatin A (TSA) was included for comparison. Sensitive cells show some arrest in G1 with hydroxamate treatment (24 hr), whereas resistant cells accumulated in G2. S phase has not been analysed. These results were confirmed independently by briefly labelling cells with ^{3}H-thymidine after drug treatment. The results showed slight inhibition of DNA synthesis in MM96L cells (52% of controls), compared with a much greater inhibition in NFF (3.7% of controls). In contrast, the non-selective drug butyrate gave levels of 10% and 2%, respectively.

Both long and short term treatment of cells revealed that ABHA was 100-fold more potent for inhibiting

growth than HMBA or azelaic acid. Treatment for 24 hr was about 10-fold less effective than for 6 days, but the former treatment time was further explored because of the limited exposure period anticipated *in vivo*. Compared on the basis of cell growth for 3 doubling times following a 24 hr treatment, ABHA was found to be highly selective against tumour cell lines compared with fibroblasts and melanocytes.

These results are summarised in Figure 3. Selectivity was also observed with HeLa, which although being the most resistant tumour line tested, was more sensitive than the normal cells. Our results show that ABHA and AAHA are 100 times more potent as differentiating agents *in vitro* than HMBA. We have found that ABHA is not only more potent than HMBA, but is selectively toxic to five human tumour cell lines, as well as to SV40-transformed melanocytes (37% survival at 30-100 µg/ml), compared to normal cells (melanocytes, fibroblasts; 37% at >300 µg/ml). This selectivity is in contrast to the effect of known cytotoxic hydroxamates (Brown, 1995) and of HMBA, which shows 37% survival at >1000 µg/ml for both tumour and normal cells. ABHA shows little toxicity to fibroblasts and melanocytes even at 1 mM concentration.

Table 2 summarises results obtained from a much larger number of cell types. These confirm the trend observed above, except for several melanoma cell lines (A2058, MM229) which showed some resistance to ABHA. It should also be noted that TSA was selective against a smaller range of cell lines than ABHA; AAHA, however, shows selectivity similar to ABHA but has slightly greater potency.

Table 2

Toxicity of Histone Deacetylase Inhibitors in Human Cells

CELL	CELL TYPE	D_{37}[a]			
		TSA (ng/ml)	ABHA (μg/ml)	AAHA (μg/ml)	HC-TOXIN (ng/ml)
Normal					
NFF	fibroblasts	50	>300	>100	>300
NM	melanocytes	170	196	>100	
Tumour					
MM96L	melanoma	10	14	8.0	62.5
MM229	melanoma	413	179	26	
MM418c1	melanoma	38	16	6	
MM485	melanoma		21		62.5
HT 144	melanoma	120	72	39	
MF10538	melanoma		62.5		
A2058	melanoma	163	236	>100	75
SILMEL13	melanoma		87.5		254
HeLa	cervical carcinoma	50	56	30	
A549	lung carcinoma	39	28	8.7	
H520	lung carcinoma		33		66.5
LIM 1215	colon carcinoma	30	26	10	50

Table 2 cont.

CELL	CELL TYPE	D_{37}[a]			
		TSA (ng/ml)	ABHA (µg/ml)	AAHA (µg/ml)	HC-TOXIN (ng/ml)
HT29	colon carcinoma		92		>300
CI80-13S	ovarian cancer	10	10	8.0	
JAM	ovarian cancer				
RDES	Ewing's sarcoma		17		25
WSB	Ewing's sarcoma		29		25
Colo16	squamous cell cancer		175		92
MCC26	Merkel cell cancer		150		270
Mutu	Lymphoma		3		
Transformed					
MelSV[40]	melanocyte		21		30
293	kidney	37	10	8.6	
HACat	keratinocytes		100		

[a] Dose required to reduce survival to 37% of control

Although ABHA and AAHA are of relatively low potency, the key finding is the selectivity of these compounds in killing melanoma, ovarian and cervical tumour cells, and a range of other tumour cell types without affecting growth of normal cells like melanocytes and fibroblasts. This is a novel result. The basis for this *in vitro* selectivity for tumour cells and the cellular target(s) are elaborated below.

Example 2 Expression of Pigmentation Markers and pRB

The activity of tyrosinase, a major enzyme in melanin synthesis, was assayed in MM96E and MM418c5 after 72 hr treatment, by which time activity had reached a minimum. The results are summarised in Table 3.

Table 3

CELL LINE	AGENT	DOSE (μG/ML)	ANTIGENIC EXPRESSION (% of control)		TYROSINASE ACTIVITY (% control)
			TRP-1	HMB-45	
MM96E	Azelaic acid	2,000	54	81	NT*
	HMBA	1,000	3.2	0.4	107 ± 7#
	ABHA	30	9.1	7.7	44 ± 4
MM418c5	Azelaic acid	2,000	75	47	NT
	HMBA	1,000	7.2	48	141 ± 12
	ABHA	30	27	160	86 ± 4

* NT, not tested

\# Mean and SD of triplicates

High levels of ABHA caused marked inhibition oftyrosinase activity in both cell lines. HMBA at an equitoxic concentration was less effective. Expression of the melanosomal antigens TRP-1 and HMB-45, determined by Western blotting, was greatly reduced by ABHA and HMBA, the latter being more effective than ABHA in the pigmented MM418c5 cells. Azelaic acid at an approximately equitoxic dose had little effect.

Treatment of the MM418 melanoma cell line with 100 µg/ml ABHA for 24 hr was found to increase the proportion of cells expressing the MHC class I molecule from 93% to 98%, as determined by flow cytometry. The MHC Class I molecule was that recognised by B7 antibody. This suggests that the compounds of the invention may be effective in increasing the proportion of tumour cells recognised by the immune system.

As shown in Figure 4, during the first 12 hr of ABHA treatment the level of hyperphosphorylated pRB (ppRB) was not affected, but a small amount of pRB (hypophosphorylated form) could be detected. After prolonged treatment of MM96L the ppRB was lost, but pRB remained in both ABHA- and HMBA-treated cells. The loss of ppRB was less marked in HeLa cells than in MM96L cells.

Example 3 ABHA-Induced Differentiation of RNA and Protein Synthesis

The RNA and protein requirements associated with ABHA-induced differentiation were investigated in HeLa cells. 5 x 10^4 cells were treated in a microtitre plate with 10 and 100 µg/ml ABHA for 24 hr. 10 µg/ml cycloheximide or 2 mg/ml actinomycin D was added at 0, 5, 10, 18 and 24 hr. The cells were then fixed with 5% acetic acid in ethanol, and stained with IFA. The dendritic cells in each well were counted, either manually or by image analysis on the inverted microscope, on the basis of cell shape.

There was an 80% reduction in the number of dendritic cells formed in response to ABHA in the presence of either of the inhibitors. ABHA-induced differentiation therefore is dependent on both RNA and protein synthesis, suggesting that ABHA is not acting solely as an inhibitor of cellular functions, but also induces the transcription of genes involved in differentiation.

Example 4 ABHA-induced Transcriptional Changes Determined by Reporter Genes

Cell clones stably transfected with a range of reporter constructs were used to test directly the effect of differentiating agents on specific gene promoters related to control of the cell cycle. In several instances the transcriptional effects of ABHA in sensitive MM96L cells and relatively resistant HeLa cells were also compared. The results are shown in Table 4.

Table 4

Transcriptional Regulation of Luciferase-Linked Reporter Constructs in MM96L and HeLa Cells

DNA Motif	Drug	Dose (µg/mL)	INDUCTION (% Control)*			
			MM96L		HeLa	
			6 hr	24 hr	6 hr	24 hr
p53 response	ABHA	30	73	36	61	172
	ABHA	100	68	25	76	202
	HMBA	1000	84	93	74	73
	Azelaic acid	2000	97	194	59	30
c-fos promoter	ABHA	30	101	639	31	388
	ABHA	100	103	459	34	580
	HMBA	1000	171	270	240	118
	Azelaic acid	2000	121	61	58	77
HIV LTR	ABHA	30	88	233	133	320
	ABHA	100	96	332	98	448
	HMBA	1000	121	104	113	55
	Azelaic acid	2000	99	87	77	38
SV40 promoter	ABHA	30	131	237	NT#	NT
	ABHA	100	130	352	NT	NT
	HMBA	1000	90	48	NT	NT

Table 4 (cont.)

DNA Motif	Drug	Dose (µg/mL)	INDUCTION (% Control)*			
			MM96L		HeLa	
			6 hr	24 hr	6 hr	24 hr
TRP-1 promoter	Azelaic acid	2000	88	48	NT	NT
	ABHA	30	42	32	NT	NT
	ABHA	100	41	28	NT	NT
	HMBA	1000	47	39	NT	NT
	Azelaic acid	2000	44	91	NT	NT

* Means of triplicates

\# NT, not tested

The results were confirmed with a second cell clone. ABHA treatment for 24 hr resulted in elevation of *c-fos* and SV40 promoter activities, whereas equitoxic levels of HMBA and azelaic acid had lesser effects or were inhibitory. All the compounds tested inhibited the TRP-1 promoter, after 6 hr or 24 hr treatment.

In some instances the sensitive MM96L cells gave different responses to ABHA compared to HeLa cells. MM96L showed inhibition of p53-activation in a 24 hr treatment. *c-Fos* promoter activity was strongly inhibited in HeLa but not in MM96L, following a 6 hr treatment with ABHA.

Example 5 Effect on Transcriptional Activity

a) Metallothionein Promoter

MM96L and HeLa cells were transfected by electroporation with the p294MetM3 plasmid containing the sheep metallothionein Ia promoter and β-galactosidase, followed by selection of stably transfected cloneswith hygromycin (Wong *et al*, 1994). For reporter assays, cells were seeded in microtitre plates ($5x10^4$/well) and treated next day. Medium was removed and β-galactosidase activity was measured in an ELISA reader at 570 nm using chlorophenol red galactoside as the substrate, essentially as previously described (Wong *et al*, 1994).

The zinc-induced activity of the metallothionein promoter in 6 stably transfected, mixed clones of MM96L cells (MM96L-gal) was highly sensitive to ABHA, enhancement being detected after a 5 hr treatment with 1 µg/ml of drug. The results are shown in Figure 5A. The dose response of 6 mixed HeLa-gal clones showed an inverse response, being inhibited to 40% of the control at 10 µg/ml ABHA. Similar trends were found when transiently-transfected cells were used. When MM96L-gal cells were treated with 10 µg/ml ABHA for 24 hr, washed, and then induced with zinc for 5 hr, increased activity (181 ± 13%) was also obtained, indicating that ABHA was not acting by transporting zinc into the cells. The ABHA enhancement of zinc activity was

not abrogated by exposure to the PKC inhibitors calphostin C (0.5 μg/ml) or bisindolyl maleimide (1 μg/ml).

b) SphI-containing Promoter

A more dramatic example of gene activation by ABHA and AAHA was found in association with a motif which forms part of the SV40 and mammalian gene promoters. A range of different promoter constructs showed that the *SphI* sequence (AAG CAT GC) was responsible for this activation, as illustrated in Figure 5B. Activation occurred with all histone deacetylase inhibitors tested, but was only observed in cells that over-expressed the tumour suppressor gene, p16, as shown in Figure 5C. This finding is relevant to identifying the range of mammalian genes that are activated by such drugs.

c) DNA Methylation

These compounds also inhibit the methylation of DNA, and thus provide a supplementary mechanism for regulation of gene expression in sensitive or resistant cells. The A4/4 cell line is a derivative of 293 cells, which were shown in Table 2 to be sensitive to ABHA and AAHA. A4/4 cells were transfected with a reporter plasmid that is silenced by DNA methylation. Treatment with a demethylating agent (5-azacytidine; Biard *et al*, 1992) inhibits DNA remethylation of the daughter strand after cell division, allowing recovery of the reporter activity, detected as β-galactosidase. Treatment of A4/4 cells for 40 hr gave substantial increases in reporter activity, as summarised in Table 5.

Table 5

Effect of Compounds of the Invention
on Recovery of Reporter Activity
(% of control)

Compound Dose (μg/ml)	ABHA	SBHA
1	180	NT
3	NT	241
10	390	NT
100	331	NT

NT - not tested

Example 6 Inhibition of Growth of Xenografted Melanoma *In Vivo*

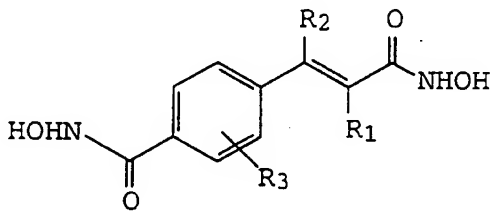
Xenografts of melanoma cell line MM96L were established in BALB/c nude (nu/nu) mice. One group of mice was treated with compounds of the invention at a dose of 4 mg/day by intraperitoneal injection. Because of limited supplies of the compounds, the dose could only be administered at 5/7 days per week. As shown in Figure 6, the growth of the melanoma was significantly inhibited by treatment with ABHA, AAHA and SBHA, compared to control, untreated mice. This is particularly noteworthy, because the melanoma cell line MM96L is resistant to treatment by conventional antitumour agents, both *in vitro* and *in vivo*. Indeed, as far as we are aware, these are the first compounds which have been demonstrated to inhibit the growth of this cell line. In contrast, TSA and HC-toxin were inactive, even though the doses used were 5-10 fold higher than the equivalent dose of ABHA.

Cultured cells from the small tumours which were still surviving at day 40 were still sensitive to the compounds, indicating that higher doses could be used. The dose administered was well-tolerated, and no signs of overt toxicity were detected. ABHA has no degradable substituents, and hydroxamates are quite stable to

metabolism *in vivo*. This is discussed further in Example 9.

Example 7 Mapping of Receptor and Optimisation of Drug Structure

A number of hydroxamate compounds have been synthesised within our group, and by others (Lofas and Johnsson, 1990). In order to map the receptor we have focused on examining the distance requirements between the two polar ends of the compounds. Thus, the carbon chain length has been varied and replaced by rigid spacers, for example aromatic rings and cinnamyl groups as in **5**.



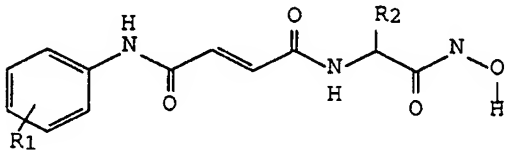
5 and derivatives

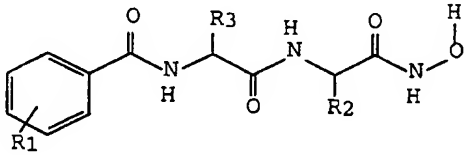
R_1 or R_2 is H, alkyl, *etc.*
R_3 is NO_2, halogen, NH_2, OH, *etc.*

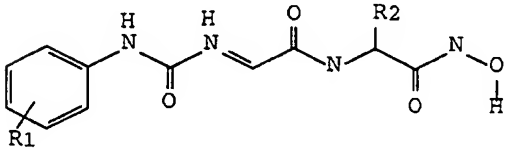
An overlay of the structures of active (grey) and inactive (white) compounds, depicted in Figure 8, clearly shows large differences in accessible three-dimensional space for these two classes. In each case, one hydroxamate moiety has been tightly superimposed while the remainder of the molecule is free to move. Active compounds form a tight cluster with the second hydroxamate (or carbonyl) in close array and the backbones tightly packed. In contrast, the inactive series forms a disparate array of second hydroxamate (or carbonyl) geometries. The key feature of this map is the relative location of the two ends of the compounds, which are in different positions for active and inactive compounds.

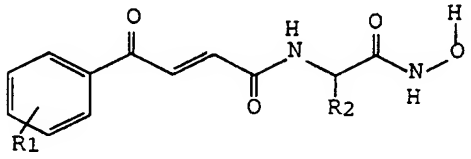
Since the initial development of this pharmacophore, significant backbone branching or substitution has been incorporated into the basic structure. A series of compounds which maintain the binding geometry of the two termini but vary in their size and substitution patterns has been synthesized in order to define the spatial requirements for receptor binding.

This aim is achieved by synthesizing both peptide-based and hydrocarbon-based compounds:





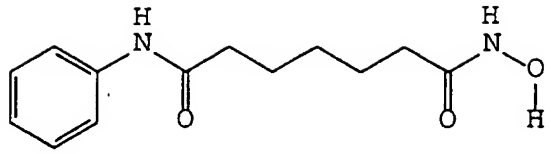




Peptide-based mono-hydroxamate libraries

R_1 = H; OH; OCH_3; Cl; Br; NO_2; Nme_2; *etc.*

R_2 and R_3 are D or L amino acid side chains



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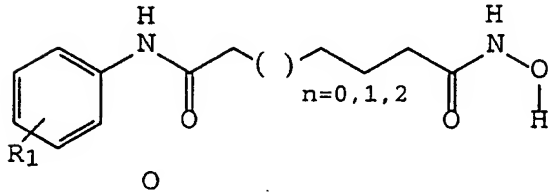
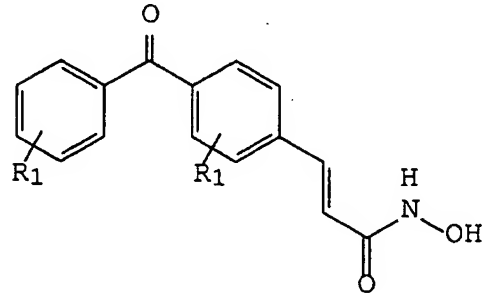
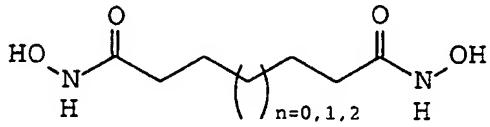
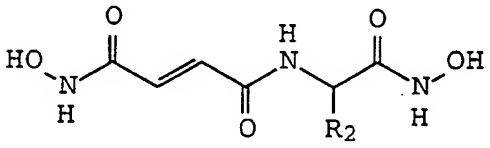


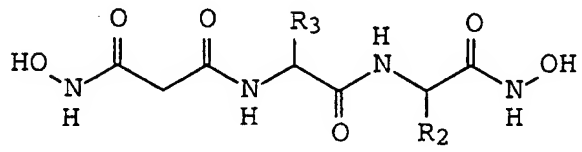


Non-peptide based mono-hydroxamate library

R_1 = H; OH; OCH_3; Cl; Br; NO_2; Nme_2; *etc.*
R_2 and R_3 are D or L amino acid side chains



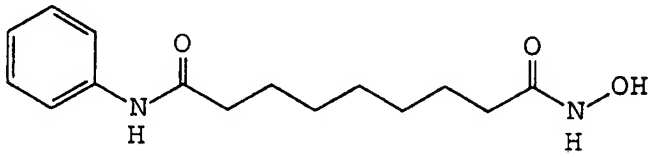




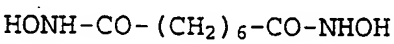
R_2 and R_3 are D or L amino acid side chains
Peptide-based bis-hydroxamate library

Preferred compounds which we have found to be active are

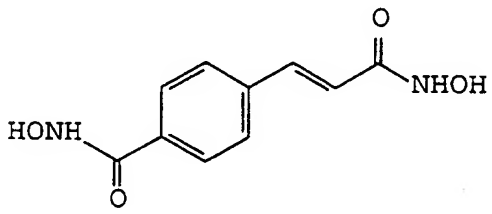
AAHA (previously designated Mk-4 in the priority application)



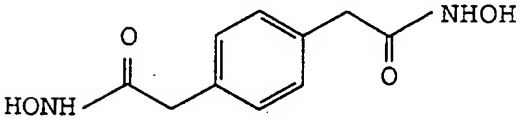
SBHA



MW2796



MW2996



The peptide-based compounds incorporate one, two or three amide bonds within the linker to act as rigid planar constraints, as well as probing hydrogen bonding interactions along the backbone of the inhibitor. In addition to these constraints, the addition of amino acid side chains (D or L) probes steric as well as hydrophobic, hydrogen bonding and charge-charge interactions within the receptor.

Example 8 Two-Dimensional Electrophoresis

Preliminary results have shown that comparison of 2-dimensional gel electrophoresis patterns, obtained as described above, can be used to generate a subtraction map to identify proteins which are present in cells treated with compounds of the invention, but are absent in untreated cells. Qualitative differences were confirmed using a subtraction map of normal NFF cells. A comparison of the two-dimensional electrophoresis patterns of treated and untreated MM96L cells is shown in Figures 7A and 7B. Samples from a number of cell lines were extracted from cytosolic proteins after 16 hr treatment with ABHA. Extracts were electrophoresed on one-dimensional gels (Figure 7C) and then samples of the MM96L lysates (control (A) and treated with 100 µg/ml ABHA (B)) were subjected to two-dimensional elecrophoresis on a pH 3-10 isoelectric focusing gel followed by an 8-18% SDS polyacrylamide gel. Increases or new proteins are shown with solid arrows, and losses are shown with white arrows.

The changes in protein expression which were observed were sufficiently large to identify bands, which are related to sensitivity, on one-dimensional protein gels, by comparison with resistant cells. This is illustrated in Figure 7C. One candidate, which we have identified as the autoantigen Ku 86 by Western blotting, is highly expressed in sensitive cells, and is further increased by ABHA treatment, as shown in Figure 7D. 25 µl of cell lysate from cytoplasmic extract or from cytoplasmic membrane and nucleus was loaded on to each lane. After Western transfer, cells were reacted with monoclonal antibody OV9D1, which reacts with Ku86 polypeptide.

This approach can generate as many as 1600 compounds for the peptidic library shown above. Initially 20-30 compounds are synthesized to probe receptor space. These focused libraries are prepared using standard solid phase peptide synthesis protocols in a multiple-pin based combinatorial chemistry approach. On the basis of initial

results obtained using these libraries, further compounds are subsequently made to optimise interactions and the pharmacological profile.

Example 9 Hydrocarbon Library

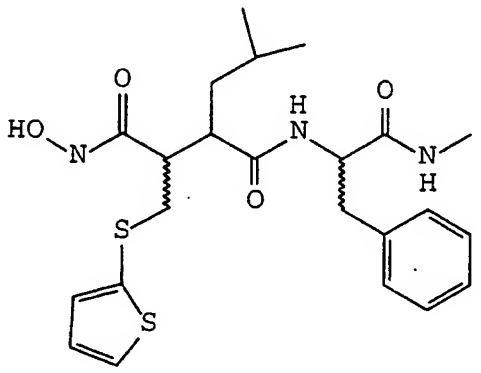
A combination of solution organic synthesis and a combinatorial non-peptide synthesis (Rockwell *et al*, 1996) may be used to prepare a hydrocarbon library.

Aromatic compounds (*eg.* **5** in Example 6) have much more rigid backbones than their peptidic counterparts. Substituents on the aromatic ring impose additional steric constraints. Whereas in the peptidic series some backbone reorganisation can relieve an unfavourable steric interaction, this is generally not the case for substituted aromatics. Consequently these latter compounds provide a more accurate but also more limited picture of steric requirements for activity. A series of up to 125 compounds (5 substituents at each of the 3 positions, R_1, R_2, R_3) was made in the first instance. These compounds were prepared by conventional solution chemistry, and characterised spectroscopically.

The requirement for two hydroxamic acid groups was also investigated. In a recent report (Richon *et al*, 1996), the monohydroxamate **6**, whose structure was shown earlier, was reported to be as effective as ABHA in inducing cell differentiation. We have tested this compound for selectivity against tumour cells over normal cells. Our initial results indicate that **6**, like ABHA, inhibits G_1 to S phase transition. In the event that **6** demonstrates selective cytotoxicity comparable to that ABHA for human cells, a series of 20-30 compounds similar to those described above, in which one hydroxamate is varied, is examined as illustrated below.

Three compound classes which are structurally similar to **6** and ABHA may be important in optimising this drug lead, for reasons described in Example 6. These are based on the monohydroxamate batimastat **7** (British

Biotechnology), which is not selective for tumour cells over normal cells when compared to ABHA.



7

These focused combinatorial libraries are used to identify salient features of the next generation of differentiating agents, and result in a 10-1000 fold increase in activity without sacrificing selectivity. Combining the information from these studies allows the development of potent and selective differentiating agents, which can be refined for improved bioavailability, and used to extract target protein(s) from cellular lysates, as discussed below.

Example 10 Evaluation of Selectivity

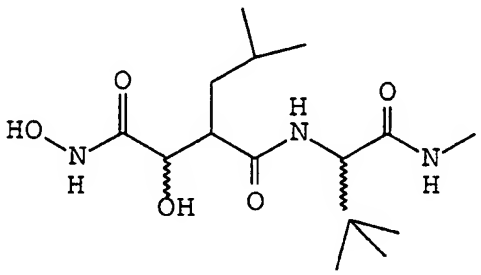
a) Cytoxicity

Comprehensive drug screening was carried out using the panel of cell lines used in Example 1. Other normal cells (neutrophils; eosinophils; macrophages; B lymphocytes), transformed cell lines and other cancer cell lines (colon and breast tumour cells) are used for further evaluation, if appropriate. These additional data enable elucidation of detailed selectivity profiles of cytotoxicity for each compound. Results like those in Example 1 are used to guide selection of candidates for *in vivo* screening.

b) Metalloprotease Inhibition

Matrix metalloproteases (MMPs) are a family of Zn/Ca enzymes that degrade the chief components of the extracellular matrix. Most malignant tumours produce high concentrations of MMPs. Although inhibited naturally by tissue inhibitors of metalloproteases (TIMPs), over-expression and activation of MMPs causes an imbalance resulting in tissue degradation. It is widely thought that MMPs are important in the growth and spread of malignant tumours, and they have also been associated with chronic diseases such arthritis and multiple sclerosis (Beckett *et al*, 1996). Although compounds such as **7** and **8** are non-selective broad-spectrum MMP inhibitors, they have been found to prevent or reduce spread or growth in models of metastasis, angiogenesis and tumour progression (Beckett *et al*, 1996). Administration i.p. of **7** was effective against malignant ascites that formed in the peritoneal cavity in a murine xenograft model of human ovarian carcinoma. However, these compounds inhibit many metalloenzymes, and are cytotoxic to at least some normal cells as well as a certain tumour cells.

The compounds of the invention are tested in metalloprotease assays, because of the known antitumour properties of hydroxamates like **7** and the more promising **8** (Beckett *et al*, 1996).



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No significant inhibition of MMP activity has been observed for any compounds of the invention.

c) Inhibition of Histone Hyperacetylation

The fundamental unit of the eukaryotic genome is the nucleosome, which is composed of DNA wrapped around a histone octamer. Histones are reversibly acetylated on the ε-amino group of Lys residues. Since interactions between acetylated histones and DNA are thought to be crucial for gene expression (Wolffe, 1996), regulators of histone acetylation might be expected to affect transcription.

Recent genetic, biochemical and immunological evidence suggests that histones involved with transcribed genes are more highly acetylated than histones from non-transcribed regions (Taunton *et al*, 1996). Inhibitors of histone deacetylase would increase the levels of histone acetylation. At concentrations in the millimolar range the weak differentiating agent, butyrate (**1**), is known to inhibit the acetylation of histones (Kijima *et al*, 1993), inducing intracellular accumulation of hyperacetylated histones. Differentiation and cell cycle arrest could possibly be attributed to hyperacetylation of histones.

Histone hyperacetylation was examined by PAGE as described above, using MM96L melanoma cells. The results are illustrated in Figure 9, and Figure 9A shows that acetylation of histone H4 is increased following exposure of the cells to ABHA at a dose of 100 µg/ml for 24 hr. We believe that this results from inhibition of histone deacetylase.

The time course (Figure 9B) shows that acetylation increases within 2 hr of treatment, and then decays over the next 24 hr if drug is removed (Figure 9C). Repeated experiments did not find any major difference between hydroxamate-sensitive and -resistant cells, as shown in Table 6.

Table 6

Acetylation of Histone H_4 During 24 hr Treatment with Differentiating Agents

Cell	TETRA-ACETYL-H_4 (% total H_4)[a]			
	O	ABHA (10 µg/ml)	ABHA (100 µg/ml)	Butyrate (5 mM)
MM96L	0	0	28±4.3[b]	18
MM229	0	0	23	13
HeLa	0	0	32	12
NFF	0	0	20±1.6[b]	22
Melanocyte	0	1.7	13	NT

[a] Histones separated on Triton-urea gels were stained with Coomassie-blue and quantitated with a laser

[b] Mean and SD of 2-3 experiments

Example 11 Low Expression of Full Length RbAp48 in Hydroxamate Sensitive Cells

Western blotting of cell lysates showed that hydroxamate-sensitive cells expressed a low molecular weight form of a protein able to bind to retinoblastoma protein, designated protein RbAp48, as shown in Figure 10A, and/or expressed low levels of the full length RbAp48, as shown in Table 7.

Table 7

Relationship Between Resistance to Histone Deacetylase Inhibitors and Expression of RbAp48

CELL	LEVEL OF IMMUNOREACTIVITY WITH RbAp48 ANTIBODY[a]	
	48 kDa	38 kDa
Resistant		
NFF	1.0	0
Melanocytes	1.01	0
MM229	2.08	0
A2058	1.60	0
Sensitive		
MM96L	0.62	0
MM418c1	0	0
HT144	0	2.45
HeLa	0	1.0
A549	0	1.65
LIM1215	0.15	0
CI80-13S	0	0.18
293	0	0.49
HACat	0	0

[a] Densitometer values of bands on Western blots, relative to the 48 kDa band obtained from an equal protein loading of NFF.

A variety of different tumour cell lines was tested. The association with sensitivity was confirmed in cell hybrids between sensitive and resistant cells, which expressed the RbAp48 pattern of the sensitive partner (Figure 10B), and were sensitive to killing by ABHA. Thus we have shown that hydroxamate sensitivity is a dominant negative phenotype in human tumour cells.

Example 12 Anti-tumour Activity *In Vivo*

The *in vivo* protocol described in Example 5 was used to test the efficacy of compounds of the invention at different doses against xenografts in nude mice of the human melanoma cell line (MM96L), an ovarian cancer cell line (JAM), a cervical cancer (HeLa), and the B16 mouse melanoma in C57 mice. The results of these initial experiments have been used for comparative evaluation of derivatives which are expected to be much more potent and selective, in order to identify compounds with better *in vivo* profiles of activity than ABHA. Some choices are made about which compounds are the most appropriate to test, based on LogPs, (octanol solubility divided by water solubility) of between 0 and 4, and likely metabolism and toxicities. Detailed dose response curves are obtained for the most promising compounds. The most active compounds are also tested in combination therapy with known antitumour agents for synergy, and cross resistance.

We have shown that the lack of activity of TSA and HC-toxin *in vivo* correlates with inactivation of their killing effects when incubated with cultured cells. This is illustrated in Figure 11. Since liver and kidney metabolism is expected to be far more active than that of the cultured cells used here, these drugs are unlikely to be effective *in vivo* even at higher doses. In contrast to this finding, compounds useful in the method of the invention, exemplified by ABHA, are both stable *in vitro* (Figure 11) and active *in vivo* (Figure 6).

Example 13 Optimisation of the Pharmacological Profile

a) Cell Uptake

In order for compounds to show a therapeutic effect, they need to penetrate cell membranes. Partition coefficients (LogP = % octanol soluble/% water soluble) are predicted during the design phase using computer software. LogP values are then experimentally determined by reverse phase HPLC. Generally, compounds desirably have

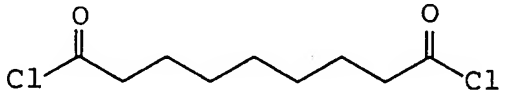
LogP values of 0 to 4, or have substituents that are known to facilitate uptake (*eg.* by amine pumps, etc).

b) Bioavailability

Compounds which are found to be sufficiently potent and selective *in vitro*, and show promise as antitumour agents *in vivo* in mice, are evaluated for bioavailability in animals. A single dose of drug (10 mg/kg) is administered i.v. and p.o. in parallel experiments to 200-250g Sprague-Dawley rats. Serum is sampled and the parameters $t_{1/2}$, T_{max}, C_{max}, F% are then calculated. For very promising candidates, further tests are performed in dogs, using similar methods.

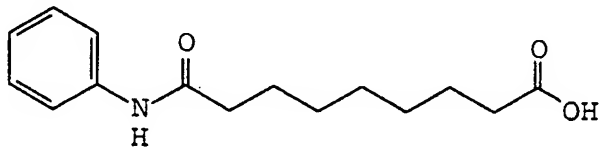
Example 14 Synthesis of the Non-Peptidic Inhibitor AAHA

To a 500 mL round bottom flask containing azelaic acid (20 g, 106 mmol) was added 25 mL of thionyl chloride. An air condenser was fitted and the mixture brought to reflux for 30 minutes after which the excess thionyl chloride was removed *in vacuo*, leaving a residue of the diacid chloride:

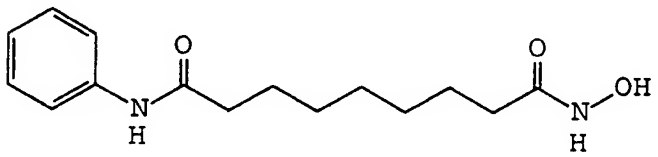


To a 500 mL round bottom flask containing the diacid chloride prepared above was added dropwise a solution containing aniline (10.65 mL, 117 mmol), N-methylmorpholine (12.85 mL, 117 mmol) and dichloromethane (100 mL). After addition was complete, the mixture was allowed to stir at room temperature for 20 minutes. The dichloromethane was then removed *in vacuo* and the residue taken up into 200 mL of 5% sodium hydroxide and extracted with 3 x 100 mL aliquots of ethyl acetate. The basic layer was removed, acidified with 6M HCl and extracted with 3 x 100 mL aliquots of ethyl acetate. The ethyl acetate

extracts were combined and dried ($MgSO_4$) and concentrated to yield the anilide. The crude product 1.93 g (6.9%) was used in the next step without further purification. NMR and mass spectral anaylsis were consistent with the desired product:



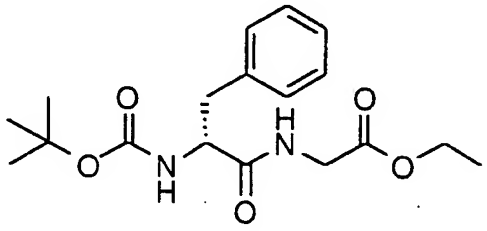
To a 50 mL round bottom flask containing the anilide (200 mg, 0.76 mmol) dissolved in dry THF (20 mL) was added N-methylmorpholine (0.092 mL, 0.85 mmol) and isobutylchloroformate (0.110 mL, 0.85 mmol). The mixture was allowed to stir for 10 minutes, after which time a solution containing hydroxylamine hydrochloride (58.2 mg, 0.85 mmol), ethanol (10 mL) and 5% sodium hydroxide (0.94 mL) was added. After a further 10 minutes the THF was removed *in vacuo* and the residue taken up into 100 mL of 1M HCl and extracted with 3 x 30 mL aliquots of dichloromethane. The organic extracts were combined, dried ($MgSO_4$) and concentrated, yielding 130 mg of crude material, which was purified by reverse phase HPLC to give 15 mg (7.1%) of the desired hydroxamic acid:



Example 15 Synthesis of Peptidic Analogue (A)

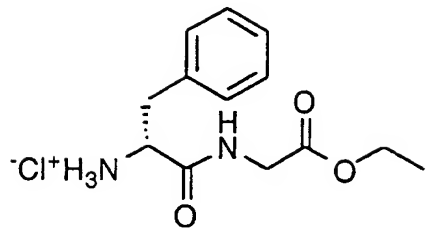
To a stirring solution of Boc-Phenylalanine (1g, 3.77mmol) and glycine ethyl ester hydrochloride (1.16g, 8.29mmol) in dichloromethane (20mL) was added benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (1.84g, 4.17mmol) followed by N-methylmorpholine

(0.796mL, 8.29mmol). After 15 minutes the mixture was added to a 100mL separating funnel and extracted with 3 x 30mL 1M HCl and 3 x 30mL saturated sodium bicarbonate. The organic layer was run through a plug of silica gel, dried ($MgSO_4$) and concentrated to yield the protected dipeptide 910mg (81.3%) which was carried through to the next step without further purification. NMR and mass spectral analysis was consistent with the desired product:



To a stirring solution of the deprotected dipeptide (433 mg, 1.51 mmol) as prepared above and p-chlorobenzoic acid (521 mg, 3.33 mmol) in DMF (3 mL) was added BOP (1.47 g, 3.33 mmol) followed by N-methylmorpholine (0.725 mL, 7.55 mmol). After 60 minutes the DMF was removed *in vacuo*, and the residue dissolved in dichloromethane (40 mL) and extracted with 3 x 30 mL 1M HCl and 3 x 30 mL saturated sodium bicarbonate. The organic layer was run through a plug of silica gel, dried ($MgSO_4$) and concentrated to yield the acylated dipeptide 574mg (97%) which was carried through to the next step without further purification. Mass spectral evidence confirmed the presence of the desired product.

The protected dipeptide (530 mg, 1.51 mmol) as prepared above was stirred at room temperature with dioxane/HCl for 30 minutes. The dioxane/HCl was then removed *in vacuo* and the crude, deprotected dipeptide carried through to the next step without further purification. Mass spectral analysis confirmed successful deprotection.



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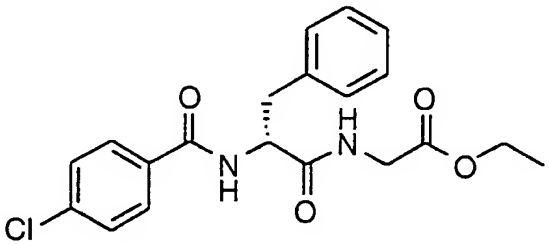
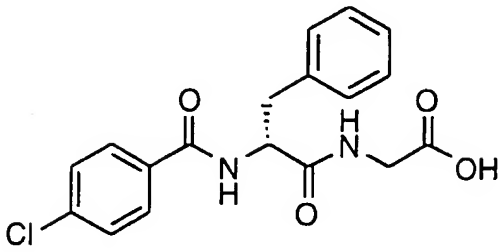
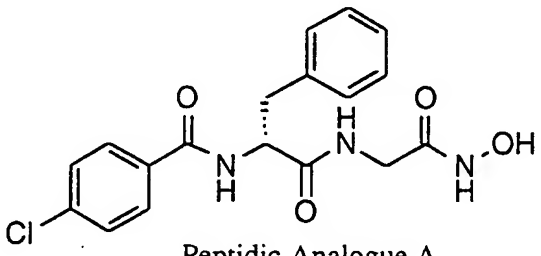


The acylated dipeptide (574 mg, 1.49 mmol) as prepared above was allowed to stir in a solution of 50% THF/water containing lithium hydroxide (125 mg, 2.97 mmol) for 30 minutes. The THF was then removed *in vacuo* and water (40 mL) added to the residue. Following acidification with concentrated HCl, a white precipitate was filtered, dried and carried through to the next step without further

purification. Mass spectral evidence confirmed the presence of the free acid:



The acid (337 mg, 0.934 mmol) as prepared above was dissolved in dry THF 5 mL containing N-methylmorpholine (0.113 mL, 1.03 mmol) and isobutylchloroformate (0.133 mL, 1.03 mmol) and allowed to stir for 15 minutes. Following this, a solution containing hydroxylamine hydrochloride (130 mg, 1.87 mmol), THF (5 mL) and 5% sodium hydroxide (1.5 mL) was added in one shot and the resultant mixture allowed to stir for a further 15 minutes. The THF was then removed *in vacuo* and water (20 mL) added to the residue. Following acidification with 1M HCl, a white precipitated was filtered which, following flash chromatography (20% dichloromethane/ethyl acetate), yielded the desired peptidic analogue A. The structure and purity of the product were confirmed by NMR and mass spectrometry.



Peptidic Analogue A

Example 16 Antiparasite Activity

The activity of the compounds of the invention against unicellular parasites of three different types was examined. The organisms selected were *Giardia duodenalis*

(also known as *Giardia lamblia*), *Trichomonas vaginalis* and *Plasmodium falciparum*.

Giardia duodenalis organisms were grown in TYI/S culture medium supplemented with 10% FCS, with and without drugs at various concentrations. One of the *Giardia* strains, WBIB-M3, was a metronidazole-resistant strain, and was maintained in medium containing 36 μM metronidazole. The minimal lethal dose (MLC) was assayed as the lowest concentration of drug at which no live parasites were present when the cultures were maintained for several days. The results are shown in Table 8.

Table 8

Activity of Differentiating Agents Against
Giardia duodenalis and *Trichomonas vaginalis* in Culture

Strain	**ABHA**	**AAHA**	**TSA**	**MW2796**	**MW2996**	**Metronidazole**
Giardia BRIS/83/HEPU/106	200*	>100	0.2	35	350	-
Giardia WB-1B	200	-	-	-	-	<100
Giardia WB1B-M3#	100	-	-	-	-	>>100
Trichomonas BRIS/92/STD/F1623#	300	-	-	-	**>350**	-

* Doses are given in µg/ml, and are the minimal lethal dose (MLC)

\# Drug resistant strains maintained on metronidazole

\- Not tested

It is evident from the table that ABHA was effective against all three strains of *Giardia* and against *Trichomonas*, with the MLC being even less for the metronidazole-resistant strain than for the wild-type strains. Of the other compounds of the invention tested, AAHA had an MLC of the same order of magnitude as that of ABHA, while that for MW2996 was higher. However, the MLC for MW2796 was much lower. TSA, which was used as a further comparison, had a particularly low MLC.

To test *in vivo* activity against *Giardia*, three day old suckling mice were injected with *Giardia duodenalis* strain 106, using a dose of 10^5 trophozooytes in 15 µl PBS pH 7.3 Mice were given drugs orally on days 7 and 8 after injections, using 250 µg of ABHA or SBHA, and 265 µg of metronidazole. *Giardia* were harvested at day 9 for the controls and day 10 for the test mice. Small intestines of mice infected with *Giardia* were excised, stripped longitudinally, kept in ice-cold PBS for 30 minutes, vortexed vigourously and parasites then counted by haemocytometer. As shown in Table 9, both ABHA and SBHA were effective at killing the *Giardia* parasites in the gut. In view of the fact that the dose and treatment regimen was probably not optimal, these agents compared more than favourably with metronidazole, which is conventionally used in therapy against these parasites.

Table 9

Treatment of Mice to Protect Against *Giardia duodenalis*

Treatment	**Number of Parasites Recovered from the Gut Measured on Day 10**
Controls	$2.8 \pm 3.88 \times 10^5$/ml
metronidazole	$3.56 \pm 2.26 \times 10^5$/ml
SBHA	0/ml (estimate of detection level $<5 \times 10^5$/ml)
ABHA	$6.67 \pm 6.24 \times 10^3$/ml

To assess activity *in vitro* against *Plasmodium falciparum*, the causative organism of the most severe form of malaria, serial dilutions of malaria trophozooytes were grown in red blood cells in the presence and absence of drug and in the presence of ^{3}H-hypoxanthine for 48 hr at 37°C. In one group of experiments, parasites resistant to conventional therapy with pyrimethamine were used. The red cells were then harvested, and the percent inhibition of parasite growth calculated from the degree of ^{3}H-hypoxanthine incorporation. The results are shown in Table 10. The values represent the amount of drug required to kill 50% of the parasite, when compared to untreated controls.

Table 10

Activity of Differentiating Agents Against *Plasmodium falciparum*

DRUGS	STRAIN		
	Normal		Drug Resistant
	Expt 1	Expt 2	
TSA	35 ng/ml	15 ng/ml	35 ng/ml
Butyrate	0.8 mM	2.5 mM	
HMBA	1.25 mM		
AAHA	<2.3 µg/ml	1 µg/ml	10 µg/ml
ABHA	<7 µg/ml	7 µg/ml	7 µg/ml
SBHA		1.5 µg/ml	1.5µg/ml
Parachlorobenzoyl-L-phenylalanine-glycine-hydroxamic acid		40 µg/ml	30 µg/ml
Salicylic Hydroxamate		75 µg/ml	70 µg/ml
MW2796		3 µg/ml	2 µg/ml
MW2996		>100 µg/ml	>100 µg/ml
Desferrioxamine		30 µM	10 µM
Pyrimethamine		0.025 µM	> 0.05 mM

It is clear that malaria parasites were also highly sensitive to the compounds of the invention. Again it can be seen that strains resistant to conventional anti-malarial agents remained sensitive to the compounds of the invention. The best effect with a drug stable in cell culture was seen with SBHA. Although TSA, which was also used as a comparison, was effective in the ng/ml range *in vitro*, it would be expected to be inactive *in vivo*, as found in the anti-tumour studies.

To test *in vivo* efficacy, the compound of the invention giving the best result in Table 10, SBHA, was tested for its ability to protect mice against infection with *Plasmodium falciparum*. Mice were injected i.p. with a mixture of rings, trophozooytes and schizonts (10^6 in 200µl PBS) and then two hours later separate groups were given 4 mg SBHAor 0.2 mg chloroquine in 0.5 ml PBS, or PBS alone (control). A second set of groups was given SBHA at the same dose at 48 hr after infection. Treatment was continued twice daily at 12 hr intervals for 3 days in all groups, and parasitaemia was scored at day 10, when the control mice had to be euthanased. The results are shown in Table 11. These clearly show that SBHA given either 2 hr or 48 hr after infection had efficacy comparable to that of chloroquine, a very potent conventional anti-malarial agent.

Table 11

Protective Activity Against *Plasmodium falciparum* in Mice

Group	**Percentage Parasitaemia in Red Blood Cells***
Controls	12.6
Chloroquine	0.2
SBHA 2 hr post-infection	0.3
SBHA 48 hr post-infection	0.2

DISCUSSION

ABHA was considerably more potent against human melanoma cells than HMBA. More surprising, however, was the high degree of selectivity for tumour cells compared with normal cells. This difference did not result simply from differences in the rate of cell cycling.

A wide range of tumour cell types was sensitive to ABHA and AAHA, indicating that differentiation mechanisms, which are tissue-specific, are unlikely to be major targets for these agents. Since activity was demonstrated against transformed keratinocytes (Colo 16 and HAcat), the invention is applicable to the treatment of conditions involving hyperproliferating keratinocytes, such as psoriasis and solar keratoses and the like.

Furthermore, the ABHA-sensitive tumour line MM96L showed major differences from a resistant line in the transcriptional activation of certain genes, particularly metallothionein and SphI.

Enhanced dendritic morphology, as shown in Figure 1, was the only evidence found for induction of differentiation by ABHA. For melanoma cells, this may relate to their neural crest origin. In the pigmentation pathway, loss of the TRP-1 and HMB-45 proteins and a decrease in tyrosinase activity indicated that ABHA acted as a dedifferentiating agent. That this occurs at least in part at the transcriptional level was suggested by loss of TRP-1 reporter activity and, in previous studies of HMBA (Sturm *et al*, 1994; Vijayasaradhi *et al*, 1995; Vijayasaradhi *et al*, 1991), loss of TRP-1 message and protein. The SV40 promoter/enhancer responds to TPA, and thus an AP-1 site in this construct may be affected by ABHA. The variety of response elements in these promoters and in the HIV-LTR sequence (Edwards, 1994), coupled with lack of specificity for activation in sensitive cells, indicates that ABHA may exert many effects which are not associated with cell selectivity.

Some primary target(s) of the compounds of the invention have been identified. A range of possibilities needs to be considered. Hydroxamic acids have the ability to chelate zinc and other metal ions, but matrix metalloproteases are not inhibited by these compounds. Furthermore, the metallothionein promoter response did not result from assisted uptake of zinc from the culture medium, because treatment with ABHA before zinc induction was also effective. The metallothionein reporter activation by ABHA in melanoma cells may result from changes in chromatin structure, as suggested for the much less potent differentiating agent butyrate (Liu *et al*, 1992), enhancing transcription of a range of genes including *c-fos*, but repressing TRP-1 and genes activated by p53. Demethylation of DNA enhances metallothionein promoter activity in certain cells (Biard *et al*, 1992), but was precluded in this study by the rapid transcriptional response to ABHA and the insensitivity of this response to 5-azacytidine. The metallothionein promoter response parallels the survival difference between HeLa and melanoma cells, and, while not necessarily involved in selective inhibition of cell growth, may help to identify the types of molecules targeted by the compounds of the invention. This promoter contains GC-rich motifs resembling Sp-1 binding sites; thus one or more steps in such a signal transduction pathway may be aberrant in melanoma cells. Metallothionein itself may play several roles in cellular signalling, including metal ion homeostasis (Hamer, 1986) and regulation of PKC (Ou and Ebadi, 1992), perhaps leading to alteration in regulators of the cell cycle.

As found in murine erythroleukemia cells treated with HMBA (Kiyokawa *et al*, 1994), ABHA induced a small elevation of pRB in melanoma cells treated for 12 hr. More significantly, and consistent with the observed G1 block in MM96L cells, pRB persisted at later times whereas ppRB was lost, in contrast to murine erythroleukemic cells, where

ppRB levels increased (Kiyokawa *et al*, 1994). Cyclin-dependent kinase inhibitor p21 (WAF-1) activity is associated with pRB hypophosphorylation, and is induced by p53-dependent and -independent pathways, which may be aberrant in melanoma and other transformed cells (Vidal *et al*, 1995). Activation of other types of gene could indirectly influence the outcome of treatment. The immune response is considered to be an important aspect of defence against cancer, and we have found enhancement of expression of MHC class I molecules, an increase which, although slight, may assist in complete elimination of tumour cells.

While not wishing to be bound by any proposed mechanism for the observed beneficial effect, we have found that the compounds of the invention inhibit histone deacetylation, thereby elevating levels of acetylated histones, which can interact with DNA to promote or inhibit gene expression and modulate DNA transcription. In contrast to the disclosure of WO 93/07148 and WO 95/31977, the compounds useful in the present invention, including ABHA, have minimal capacity to induce differentiation in neoplastic cells.

It is likely that all of the compounds of the invention are structural mimics of acetylated lysine ($RNHC(COR')-(CH_2)_4-NH-COCH_3$), in various forms which enable the compounds to have high affinity for histone deacetylase(s), but render the compounds cell-permeable and resistant to the acetylation/deacetylation reaction. In particular, we find that the transcription of *SphI*-like promoter-driven genes is dramatically activated by the compounds. Such changes may be necessary for cell killing, but not necessarily for selectivity. Regarding the latter, we have shown that selective cytotoxicity to tumour cells may be facilitated by low or aberrant expression of the protein RbAp48, and by lack of cell cycle checkpoints that protect normal cells. This suggests that the compounds of this invention can be used in combination with certain

known antiproliferative drugs to enhance the tumour selectivity of the latter. We also suggest that compounds of the present invention induce the expression of effector molecules that enhance the immune response against tumours *in vivo*, such as antigens of the major histocompatibility complex, and viral antigens in virus-related tumours, and we suggest that simultaneous treatment with IL-2 will overcome the down-regulation of IL-2 expression by histone deacetylation inhibitors that has been reported by Takahashi *et al* (1996) and thus enhance the *in vivo* anticancer action of these compounds.

Xenografts of the melanoma MM96L cells were significantly inhibited by daily treatments with 4 mg/kg ABHA, AAHA or SBHA, the first drugs of any kind to show significant activity against this cell line. Although this is a relatively high dose, other differentiating agents such as butyrate have been used safely at similar levels in the clinic to treat children with sickle cell anemia. MM96L cells which were established from a metastasis, have a mutator phenotype, and are resistant to current anticancer agents *in vitro* and *in vivo*, represent a rigorous yet probably realistic model for testing new agents. Cells cultured from the small mouse tumours that survived treatment by ABHA were still sensitive to this drug. This suggests that even higher doses of ABHA could be given, since there were no overt signs of toxicity or side effects. AAHA, which was administered in DMSO because of its poor solubility in aqueous phosphate buffered saline, and SBHA had even better activity. Metabolism of TSA, detected for the first time in this study, is unlikely to explain the resistance of NFF cells to this agent, because the exposure to drug was limited to 24 hr. Thus, although TSA is widely used as a potent and specific inhibitor of histone deacetylases *in vitro*, metabolically stable inhibitors may be required in order to obtain useful activities *in vivo*.

Acetylation of histone H4 was found to be induced by ABHA and AAHA, presumably by inhibition of histone deacetylase activity, as shown previously for TSA. This could be expected to profoundly alter gene expression, and may be a necessary condition for cell toxicity to occur. However, the present comparison of acetylation and deacetylation rates in intact, sensitive and resistant cells failed to find any evidence that differences in H4 acetylation could be responsible for the differential toxicity of the compounds of the invention. It is possible that other histone modifications may be involved in selectivity, or that another type of drug target may be responsible.

We have identified RbAp48 as a protein closely associated with resistance to ABHA, both in individual cell lines and in hybrids between sensitive and resistant cells. The normal role of RbAp48 in mammalian cells has not yet been defined. The RbAp48 counterpart in yeast inhibits the Ras-cAMP pathway, possibly via inhibition of PKA. RbAp48 could also be involved in scaffolding or matrix attachment of chromatin to DNA (MAR regions), shielding genes from neighbouring elements. Since the histone deacetylase inhibitor trapoxin binds RbAp48, RbAp48 is a candidate target of the compounds of the invention. In sensitive cells, the depleted level of full-length RbAp48 and/or competition with a truncated or altered form may lead to loss of function of wild type RbAp48 and consequently, possibly in conjunction with histone hyperacetylation, may compromise the viability of cycling cells. In contrast, as indicated by the cell cycle studies and selective inhibition of DNA synthesis, normal cells are checked in G2/M until the drug is removed, and can then safely continue in the cycle. These findings justify the use of ABHA, AAHA, SBHA and their derivatives in combination chemotherapy, to provide tumour specificity to cycle-specific drugs such as antimetabolites, including cytosine

arabinoside, 5-fluorouracil, methotrexate, chlorodeoxyadenosine, etoposide, taxol (paclitaxel), and the like, by protecting normal proliferating cells, particularly in the bone marrow and gut. Regulation of apoptosis and cell division itself may be highly sensitive to the changes in nucleosomal structure and charge that result from untimely histone hyperacetylation, and to presumptive alterations in chromatin structure arising from an altered RbAp48. The same considerations may also apply to parasites.

Overall, our results suggest that in addition to greatly increased potency, the compounds of the invention have a more specific range of cellular targets than reported in the prior art for differentiating agents, resulting in selectivity for transformed and cancerous cells. Furthermore, our identification of metallothionein and *SphI* transcription activation, loss of G2/M blocking and reduction in expression of full-length RbAp48 have provided markers of this selectivity, which can be exploited in the treatment and prognosis of cancer.

The loss of contact inhibition in neoplastic and dysplastic cells and the changes in cell morphology induced by the compounds of the invention indicate that cell surface phenomena such as adhesion molecules may also play a role in their selective action.

Advantages of the compounds of the invention over previously known anti-cancer agents are:

1. Unusually high selectivity for transformed and cancerous human cells compared to normal cells.
2. Lack of inhibition of growth of normal cells, at concentrations that inhibit transformed and cancerous cells, unlike other monohydroxamates that are known to have anti-tumour activity but are non-selective cytotoxins (Wang *et al*, 1994).

3. Little if any development of resistance by the cancer cells; human melanoma cells are still sensitive to the compounds of the invention after at least 10 cycles of treatment. In our experience with most other anticancer drugs, drug resistance is developed after only a few treatments.
4. No rapid metabolism to inactive forms by cultured cells, in contrast to other histone deacetylase inhibitors.
5. Activity *in vivo* against human tumours in a well-recognized mouse xenograft model.
6. No obvious side effects in mice, even after high daily doses for 6 weeks.
7. Chemical structures that can be readily modified to obtain further derivatives.
8. Upregulation of molecules that might enhance an antitumour immune response.
8. The mechanism of action is more specific at the molecular level than current drugs, and is capable of exploiting the loss of cell cycle check points specifically in tumour cells.

Advantages of the compounds of the invention over current agents for the treatment of parasites include:

1. Lack of toxicity to normal human cells, as detailed above, with a consequent higher therapeutic margin of safety.
2. Activity against parasites, including *Plasmodium falciparum*, which have become resistant to current agents.
3. Stability *in vivo*, confirmed by *in vivo* activity.
4. No evidence of phototoxic side reactions.

It will be apparent to the person skilled in the art that while the invention has been described in some

detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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CLAIMS:

1. A method of treatment of cancer, comprising the step of administration of an effective amount of a nitrogen-containing compound, a compound structurally related thereto, or a derivative thereof, to a mammal in need of such treatment, said compound having selective cytotoxicity for neoplastic cells compared to normal cells, and having minimal or absent ability to induce differentiation in neoplastic cells,

and in which the compound additionally has one or more of the following activities:

a) inhibition of growth in cell culture of at least one of the following human tumour cell lines: melanoma MM418c1, cervical HeLa, melanoma A2058, ovarian JAM, and lymphoma Mutu;

b) inhibition of growth in cell culture of transformed keratinocytes and melanocytes (Mel-SV);

c) inhibition of growth *in vivo* of human tumour cells in xenografted nude mice;

d) inhibition of histone deacetylase, as measured by extent of hyperacetylation of histones;

e) induction of differences in protein expression by human tumour cells compared to normal human cells;

f) selective killing of tumour cells in (a) without killing normal cells;

g) blocking of cell cycle progression of some sensitive tumour cells in the G1/S phase;

h) induction of apoptosis in tumour cells; and

i) inhibition of DNA synthesis in normal but not in tumour cells.

2. A method according to Claim 1, in which the compound is a monohydroxamate or bishydroxamate compound, or a derivative thereof.

3. A method according to Claim 1, in which the compound is a cyclic peptide.

4. A method according to any one of Claims 1 to 3, in which the compound has the ability to inhibit deacetylation of histones.

5. A method according to any one of Claims 1 to 3, in which the compound additionally has the ability to enhance zinc-induced activity of the metallothionein Ia promoter, and/or activity of the *SphI*-containing promoter.

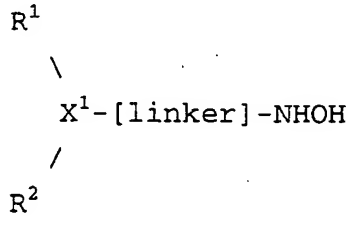
6. A method according to any one of Claims 1 to 5 that is selectively toxic to cells that express low levels of full length RbAp48.

7. A method according to any one of Claims 1 to 6, in which the cancer is a leukaemia, a lymphoma, a skin cancer, melanoma, ovarian cancer, cervical cancer, breast cancer, prostate cancer, endometrial cancer, lung cancer, gastric cancer, or colon cancer.

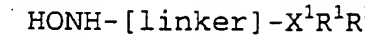
8. A method according to any one of Claims 1 to 7, in which the mammal is a human.

9. A method according to any one of Claims 1 to 8, in which the compound is not acylaic bishydroxamic acid.

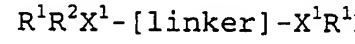
10. A hydroxamate or hydroxamic acid or derivative compound of general formula Ia , Ib or Ic:



Ia



Ib



Ic

in which X^1 is a polar group chosen from among -C=O; $-COR^1$; $-CF_2$; $-CNH_2$; $-CNR^1$; $-SO_2-$; -P(O)(OH)-; -C=S; $-CSR^1$; $-C-COR^1$; $-C-CONR^1R^2$; or $-C-CH_2OH$; or either R^1 or R^2 is absent;

R^1 and R^2 are the same or different, and each is independently selected from the group consisting of H; OH; NH_2; NHOH; substituted or unsubstituted, branched or unbranched alkyl, alkenyl, alkylamino, alkyloxy or arylalkyloxy; substituted or unsubstituted aryl, aryloxy or pyridino; substituted or unsubstituted arylamino, piperidino, cycloalkyl, cycloalkylamino, pyridineamino, 9-purine-6-amine, and thiazoleamino; and

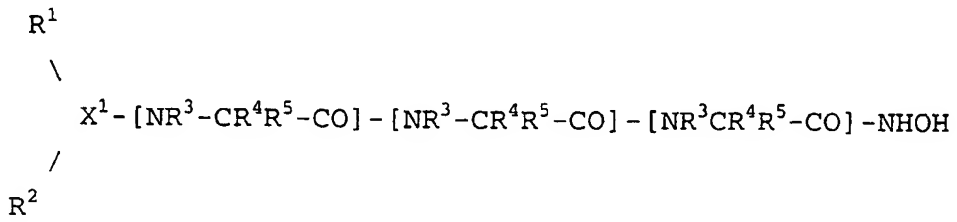
the linker is a group having a backbone of 5 to 9 atoms,

or a pharmaceutically-acceptable salt, ester or derivative thereof,

in which the compound is not azelaic bishydroxamic acid or a compound disclosed in International Patent Publications No. WO 95/31977 or No. WO 93/07418.

11. A compound according to Claim 10, in which the linker comprises one, two or three amino acids.

12. A compound according to Claim 11, in which the compound is of formula II:



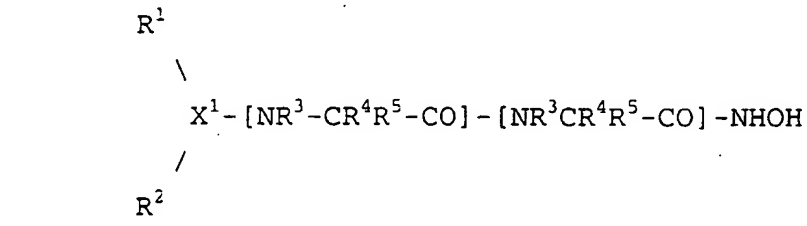
II

in which R^3 is selected from the group consisting of H; OH; NH_2; NHOH; substituted or unsubstituted, branched or unbranched alkyl, alkenyl, alkylamino, alkyloxy or arylalkyloxy; substituted or unsubstituted aryl, aryloxy or pyridino; substituted or unsubstituted arylamino,

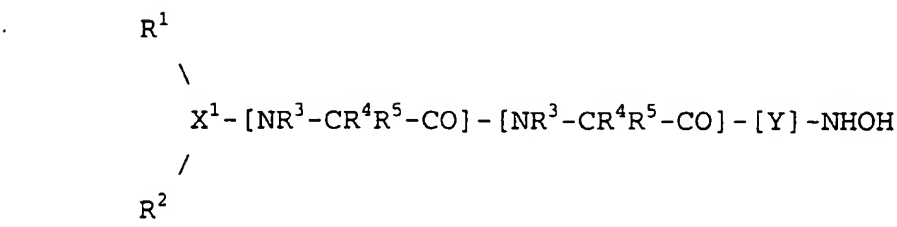
piperidino, cycloalkyl, cycloalkylamino, pyridineamino, 9-purine-6-amine, and thiazoleamino, and

R^4 and R^5 are the same or different, and is each independently selected from H, alkyl, aryl or a side-chain of a common or uncommon amino acid.

13. A compound according to Claim 10 or Claim 11, in which the linker comprises two amino acids, and the compound is of formula IIIa or IIIb:



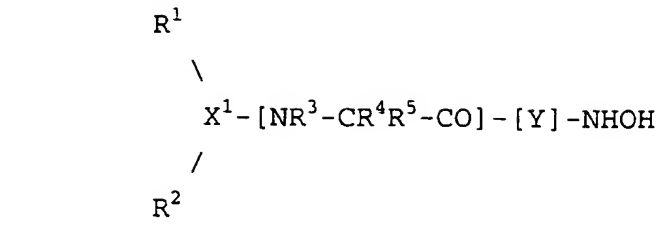
IIIa



IIIb

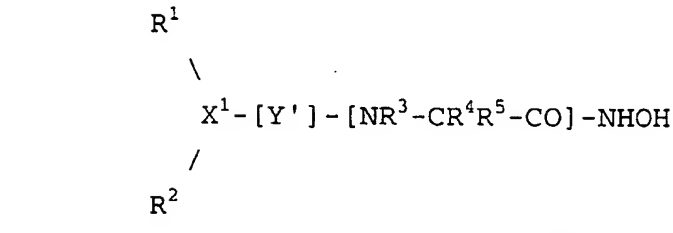
in which R^3, R^4 and R^5 are as defined in Claim 12, and Y is $-CH=CH_2-CO$; $-C(alkyl)=C(H \text{ or } alkyl)_2$; $-C_6H_4-CO$; $-CH(alkyl)-CH(alkyl)-CO$; $-NR^6CH_2CH_2CO$; $-NR^6C(alkyl)-C(H \text{ or } alkyl)-CO-$; or $-NR^6-CH_6H_4-CO$, where R^6 is as defined above for R^3, and alkyl can be a linear or branched chain aliphatic group.

14. A compound according to Claim 10 or Claim 11, in which the linker comprises one amino acid, and the compound is of general formula IVa,



IVa

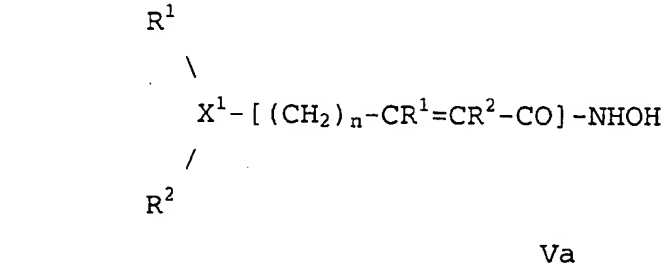
in which Y is as defined in Claim 13, and R^3, R^4 and R^5 are as defined in Claim 12, or formula IVb,



IVb

in which Y' is $-CH=CH_2-CO$; $-(CH_2)_n$, where n is an integer from 1 to 6; $-(CH_2)_3$; $-(CH_2)_4$; $-(CH_2)_2CO-$; $-(CH_2)_3-CO$; C_6H_4; $C_6H_4-CH=CH_2$; $-CH=CH_2-C_6H_4$; -CH(alkyl)-CH(alkyl); $-C_6H_4-CO$; $-C_6H_4-CH=CH-CO$; $-CH=CH-C_6H_4-CO$; or -CH(alkyl)-CH(alkyl)CO, and R^3, R^4 and R^5 are as defined in Claim 12.

15. A compound according to Claim 10, in which the linker comprises 1, 2 or 3 double bonds, and the compound is of formula Va, Vb, Vc, Vd Ve, Vf or Vg,

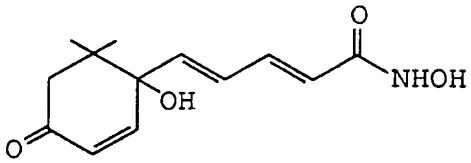


Va

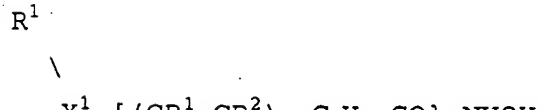
Vb

Vc

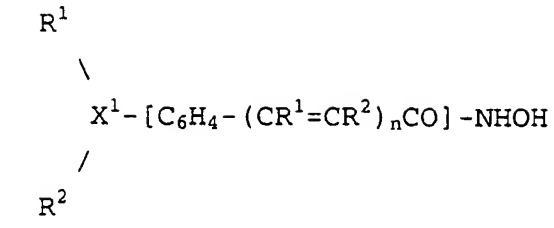
Vd



Ve



Vf



Vg

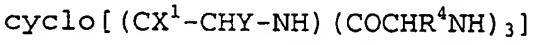
in which R1 and R2 may be the same of different, and are defined in Claim 10,

n and m is each independently an integer from 1 to 6;

and in which the C_6H_4 group is an aromatic ring, optionally substituted at the ortho-, meta- or para-position with a substituent selected from the group consisting of NO_2, NH_2, NMe_2, Cl, F, SO_2NH_2, Me and alkyl.

16. A compound according to Claim 14, in which each of R^1 and R^2 is independently H, alkyl or aryl.

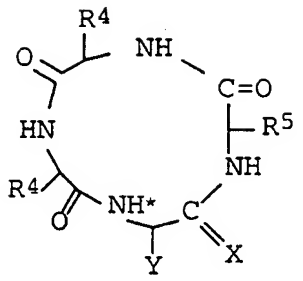
17. A compound according to Claim 10, in which the polar group X^1 forms part of a cyclic tetrapeptide of formula VI:



VI

in which R^4, X^1 and Y are as defined in Claims 9 and 10 respectively, or Y may be $(CH_2)_5COMe$, $(CH_2)_4COMe$, $(CH_2)_5CO$-alkyl, $(CH_2)_5CO$-aryl, $(CH_2)_5CO-NR^3R^6$, wherein R^3 and R^6 are the same or different, and R^6 is as defined in Claim 13.

18. A compound according to Claim 17, of general formula VII:

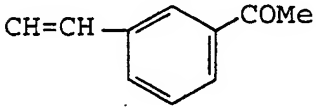


VII

in which each of R^3, R^4 and R^5 are the same or different, and are as defined in Claim 12, or may be thioproline, hydroxyproline, pipecolic acid, or decahydroisoquinoline, and

the stereochemical configuration at the position marked by * may be R or S (L or D).

19. A compound according to Claim 17, in which each of R^3 and R^6 is selected from the group consisting of H, alkyl, aryl, $(CH_2)_5CHO$;



, and $CH=CH-CH=CH-CH_2-COMe$; and

one or more of the four amino acids is optionally N-alkylated with an aliphatic alkyl group.

20. A compound according to Claim 10, in which the polar group X^1 forms part of a cyclic pentapeptide, and the compound is of formula VIII,

$$cyclo[CX^1\text{-}CHY\text{-}NH)(COCHR^4NH)_4]$$

VIII

in which X^1 and Y are as defined in Claims 10 and 12 respectively.

21. A compound according to Claim 10, which is a cyclic molecule selected from the group consisting of

quinolines, isoquinolines, tetrahydroquinolines and decahydroquinolines.

22. A compound according to Claim 10, selected from the group consisting of

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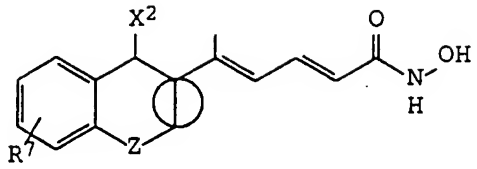
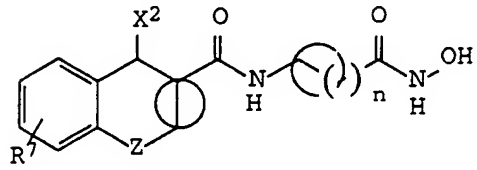
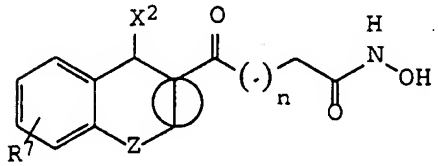
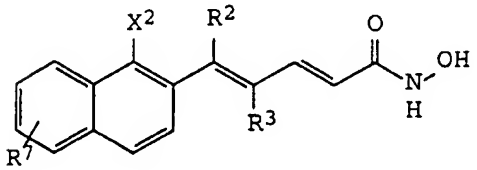


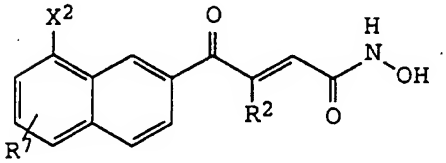


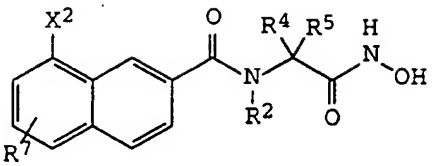
n=0,1,2

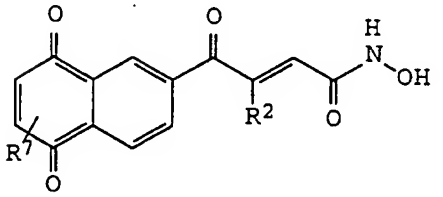


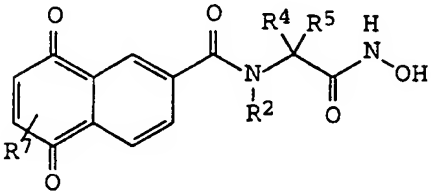
n=1,2,3

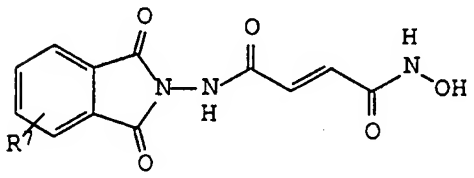


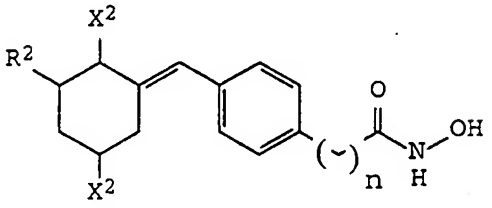




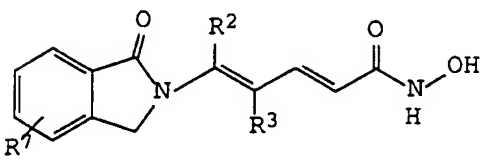


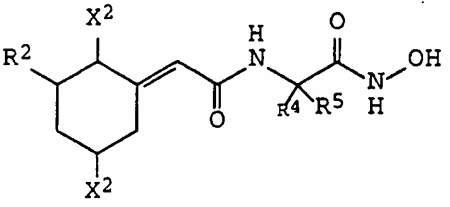


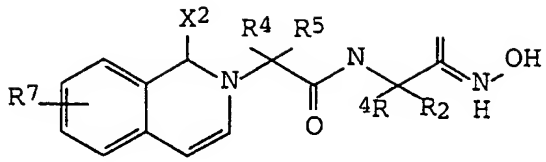


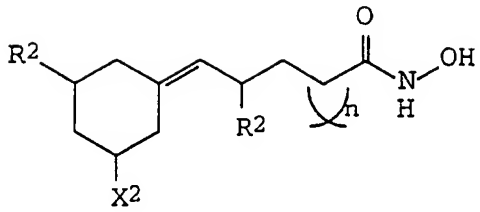


n=1.2



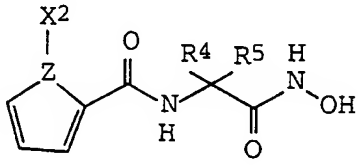






n=0,1,2

and



in which Z is O, S, NH, N-alkyl; NO; SO; CO; C-R^7;

X^2 is O, OH, aldehyde, ketone, CF_3; NO_2; NO; SH; S; NH; NH_2; CO_2H; $CONH_2$; CO_2(alkyl) or CONH(alkyl;

R^7 is one or more substituents selected from the group consisting of H; OH; OMe; NO_2; Cl; Br; F; $(Me)_2N$; CN; NH_2: NH(alkyl); $N(alkyl)_2$; SO_3H; SO_2NH_2; alkyl CF_3; O(alkyl); SH and S(alkyl), and in which

each bond depicted as an alkene bond may alternatively be a single bond, and each single bond marked with a circle may alternatively be a double bond.

23. A compound according to any one of Claims 1 to 22, which has selective cytotoxicity for neoplastic cells compared to normal cells, and has minimal or absent ability to induce differentiation in neoplastic cells, and one or more of

a) the ability to inhibit deacetylation of histones,

b) the ability to enhance zinc-induced activity of the metallothionein Ia promoter,

c) the ability to enhance activity of the SphI promoter.

d) the ability to be selectively toxic to cells that have low levels of full length RbAp48.

24. A compound according to any one of Claims 10 to 23, which is selectively toxic for two or more types of tumour cells.

25. A composition comprising a compound according to any one of Claims 10 to 24, or a pharmacologically acceptable salt thereof, together with a pharmaceutically or veterinarily acceptable carrier.

26. A method of treatment of cancer, comprising the step of administering an effective amount of a compound accordingly to any one of Claims 10 to 24 to a subject in need of such treatment.

27. A method of treatment of a protozoal parasite infection, comprising the step of administering an effective dose of the step of administering an effective dose of a compound according to any one of Claims 10 to 24, or of ABHA or a related compound as defined herein, to a subject in need of such treatment.

28. A method according to Claim 27, in which the parasite is a member of a genus selected from the group consisting of *Giardia*, *Cryptosporidium*, *Trichomonas*, *Histomonas*, *Plasmodium*, *Toxoplasma*, *Trypanosoma*, *Babesia*, *Balantidium*, *Naegleria*, *Entamoeba* and *Eimeria*.

29. A method according to Claim 28, in which the parasite is a member of the genera *Giardia*, *Trichomonas* or *Plasmodium*.

30. A method according to Claim 29, in which the parasite is *Giardia duodenalis*, or *Plasmodium falciparum*.

31. A method of treatment of a keratinous hyperplastic or dysplastic condition, comprising the step of administering an effective dose of the step of administering an effective dose of a compound according to any one of Claims 8 to 24, or of ABHA or a related compound as defined herein, to a subject in need of such treatment.

32. A method according to Claim 30, in which the condition is psoriasis, leukoplakia, or solar keratosis.

33. A method of identification of cancers which are particularly amenable to treatment by the method of any one of Claims 1 to 9, comprising the step of detecting abnormal levels or absence of full length RbAp48 in a sample of the cancer.

34. A method according to Claim 33, which comprises the step of subjecting a histological section of the tumour, obtained via biopsy or at the time of surgical excision of the tumour, to immunohistochemical analysis with an antibody directed to RbAp48.

35. A method of enhancing the selectivity of treatment of a cancer or of a parasite infection with a compound according to any one of Claims 10 to 24, or with ABHA or a related compound as defined herein, comprising the step of administering a nucleic acid sequence complementary to a nucleic, acid sequence encoding RbAp48 or an SphI-containing sequence to the subject to be treated.

36. A method according to Claim 35, in which the complementary sequence is targeted to tumour cells or to parasites.

37. A method of increasing the proportion of tumour cells recognised by the immune system, comprising the step of administering a compound according to any one of Claims 10 to 24, or ABHA or a related compound as defined herein, to a subject suffering from the tumour thereby to increase the proportion of tumour cells expressing MHC Class I molecules.

38. Use of a compound according to any one of Claims 10 to 24 in the treatment of cancer.

39. Use of a compound according to any one of Claims 10 to 24, or of ABHA or a related compound as defined herein, in the treatment of a protozoal infection.

40. Use according to Claim 39, in which the protozoal infection is caused by a parasite which is a member of a genus selected from the group consisting of *Giardia*,

Cryptosporidium, *Trichomonas*, *Histomonas*, *Plasmodium*, *Toxoplasma*, *Trypanosoma*, *Babesia*, *Balantidium*, *Naegleria*, *Entamoeba* and *Eimeria*.

41. Use according to Claim 39, in which the parasite is a member of the genera *Giardia*, *Trichomonas* or *Plasmodium*.

42. Use of a compound according to any one of Claims 10 to 24, or of ABHA or a related compound as defined herein, in the manufacture of a medicament for the treatment of a protozoal infection.

43. Use of a compound according to any one of Claims 10 to 24, or of ABHA or a related compound as defined herein, in the manufacture of a medicament for the treatment of a hyperplastic or dysplastic condition.

44. Use according to Claim 43, in which the condition is psoriasis, leukoplakia or solar keratosis.

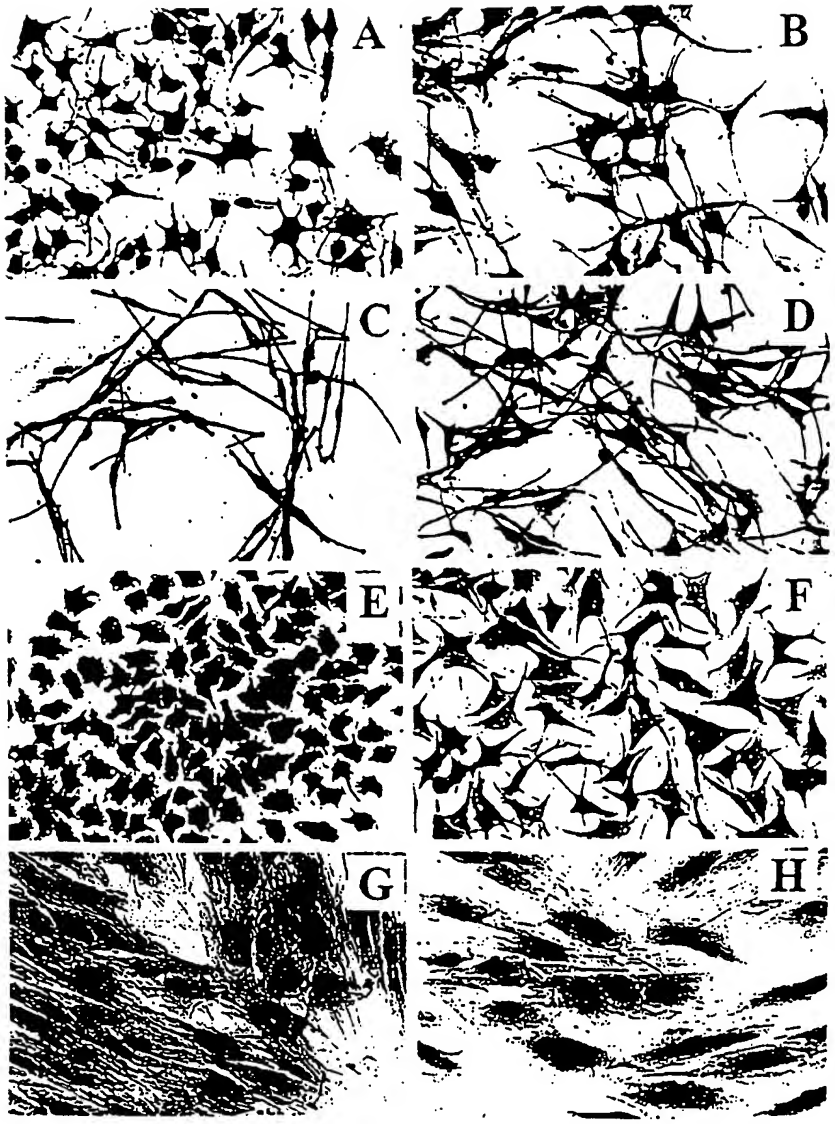


FIGURE 1A

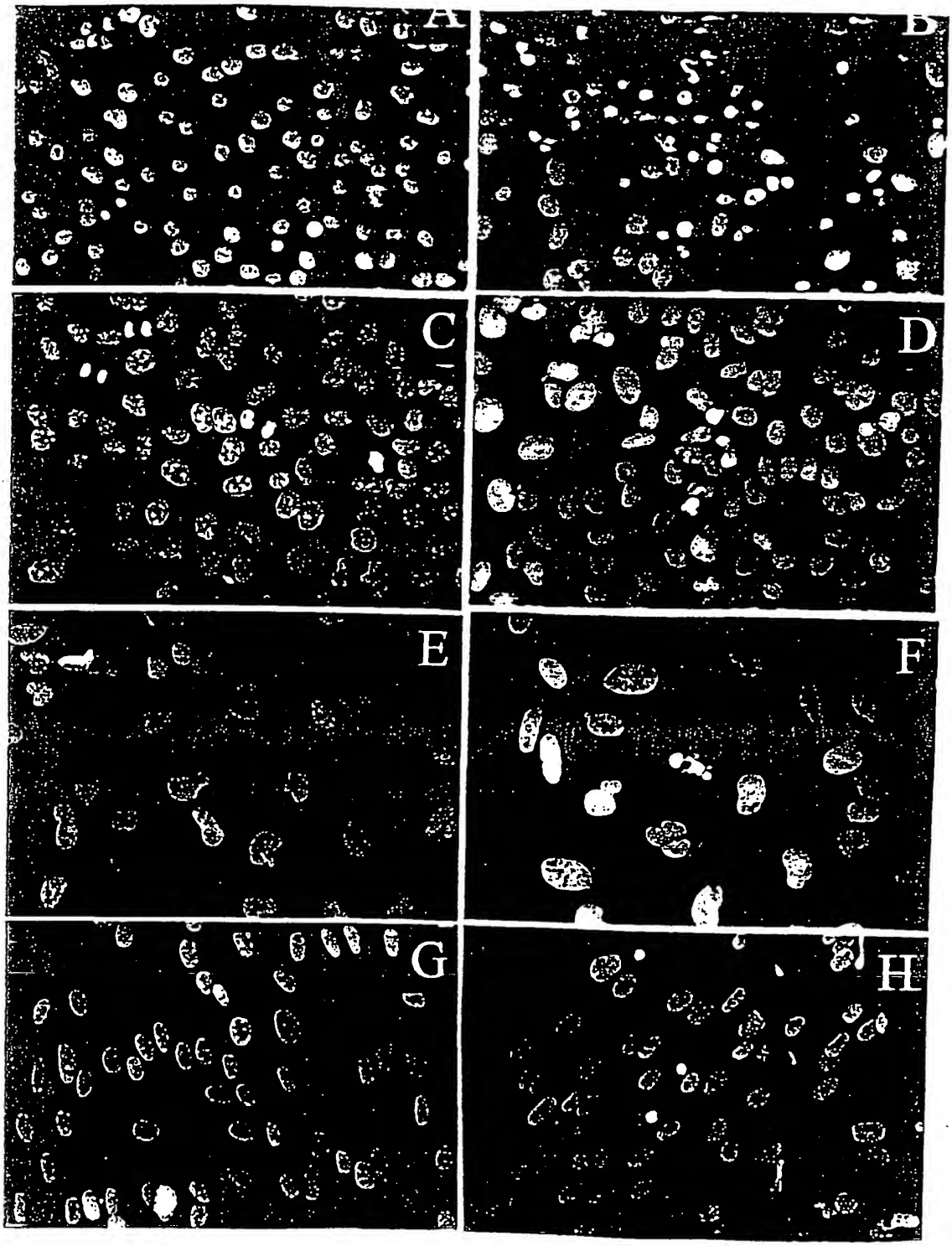


FIGURE 1B

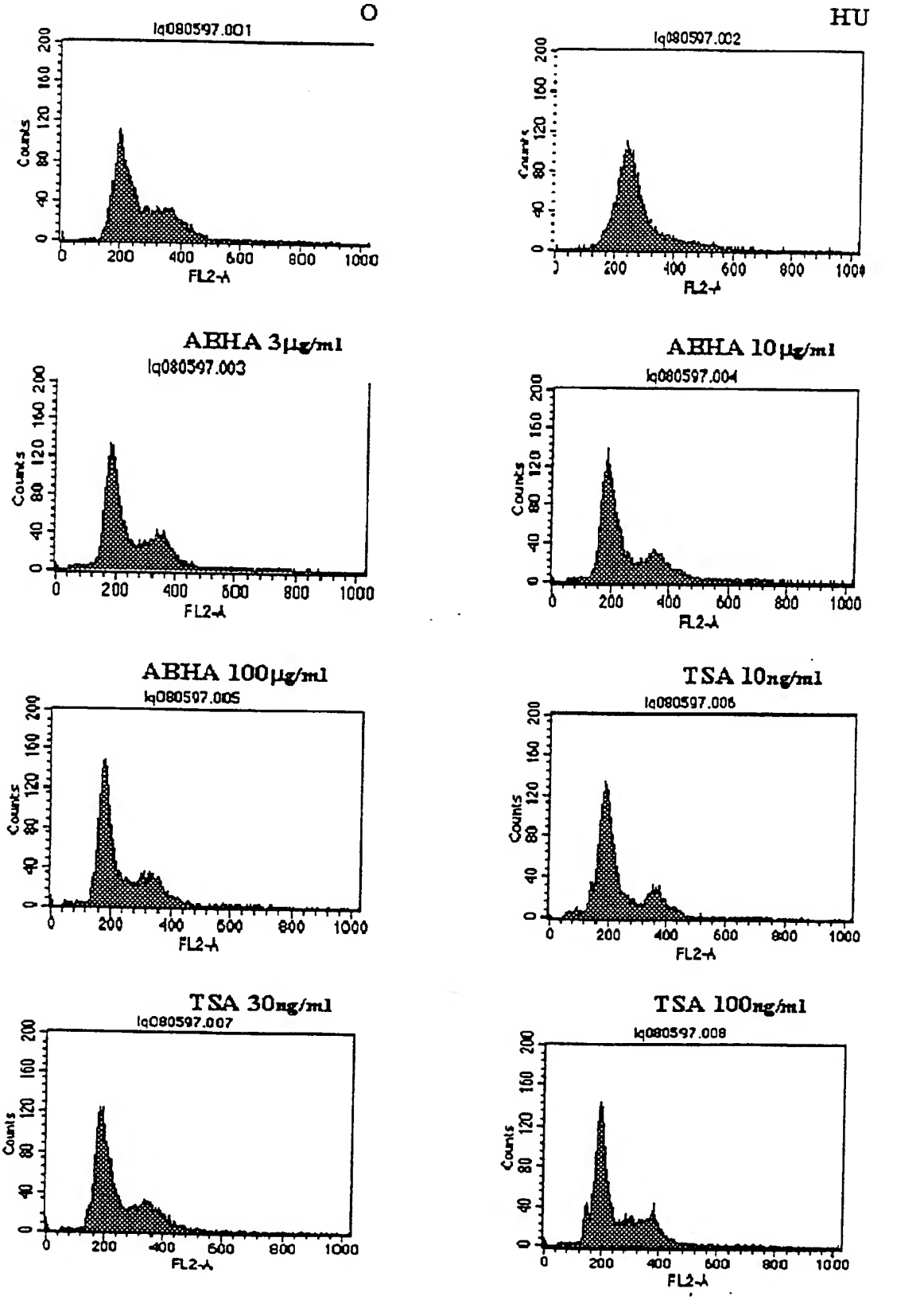

MM96L Sensitive cells
blocked in G1 and G2/M
O
HU
ABHA 3µg/ml
ABHA 10µg/ml
ABHA 100µg/ml
TSA 10ng/ml
TSA 30ng/ml
TSA 100ng/ml
lq080597.001
lq080597.002
lq080597.003
lq080597.004
lq080597.005
lq080597.006
lq080597.007
lq080597.008
Counts
FL2-A

FIGURE 2

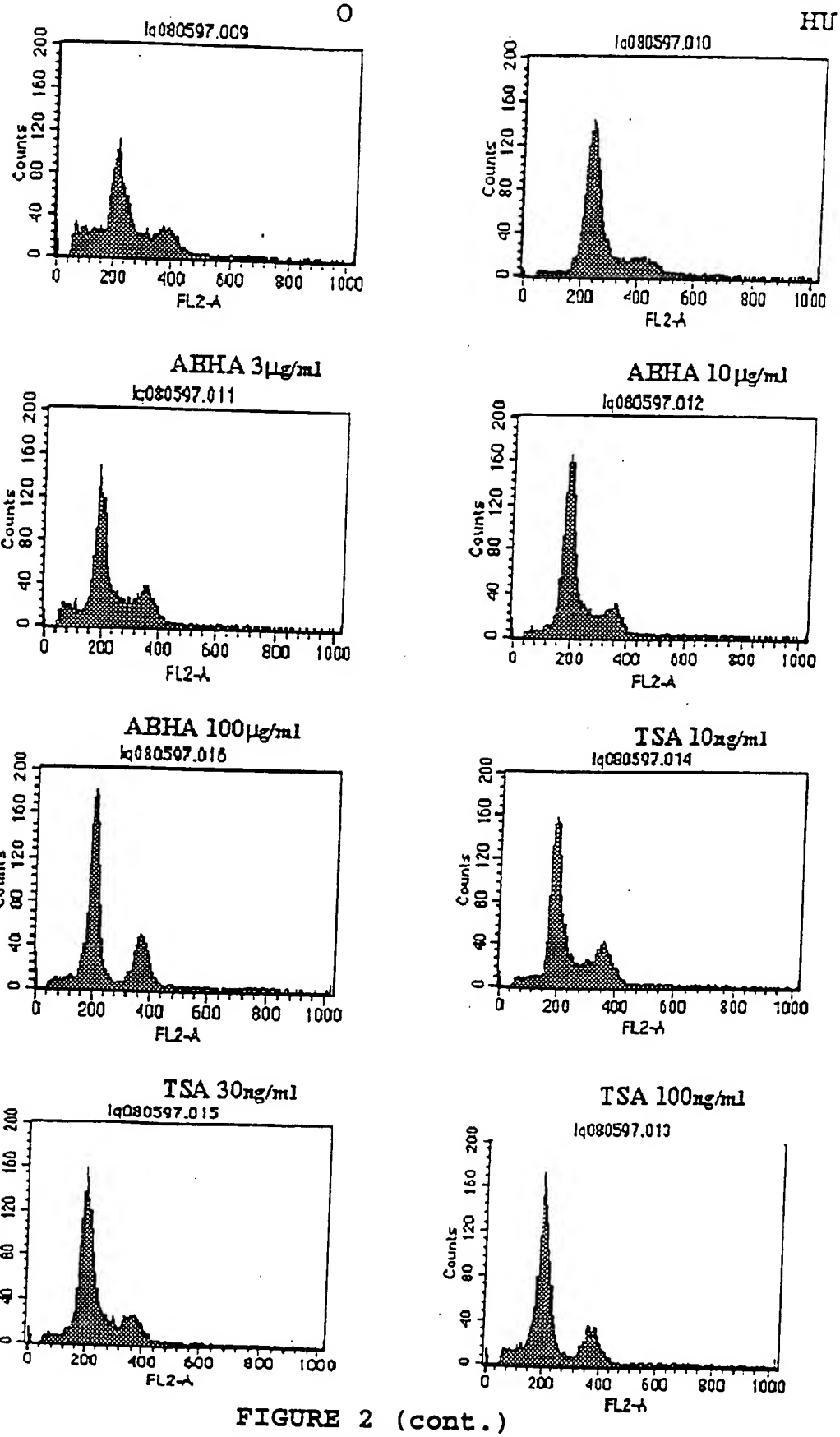


FIGURE 2 (cont.)

NFF Resistant cells blocked in G2/M

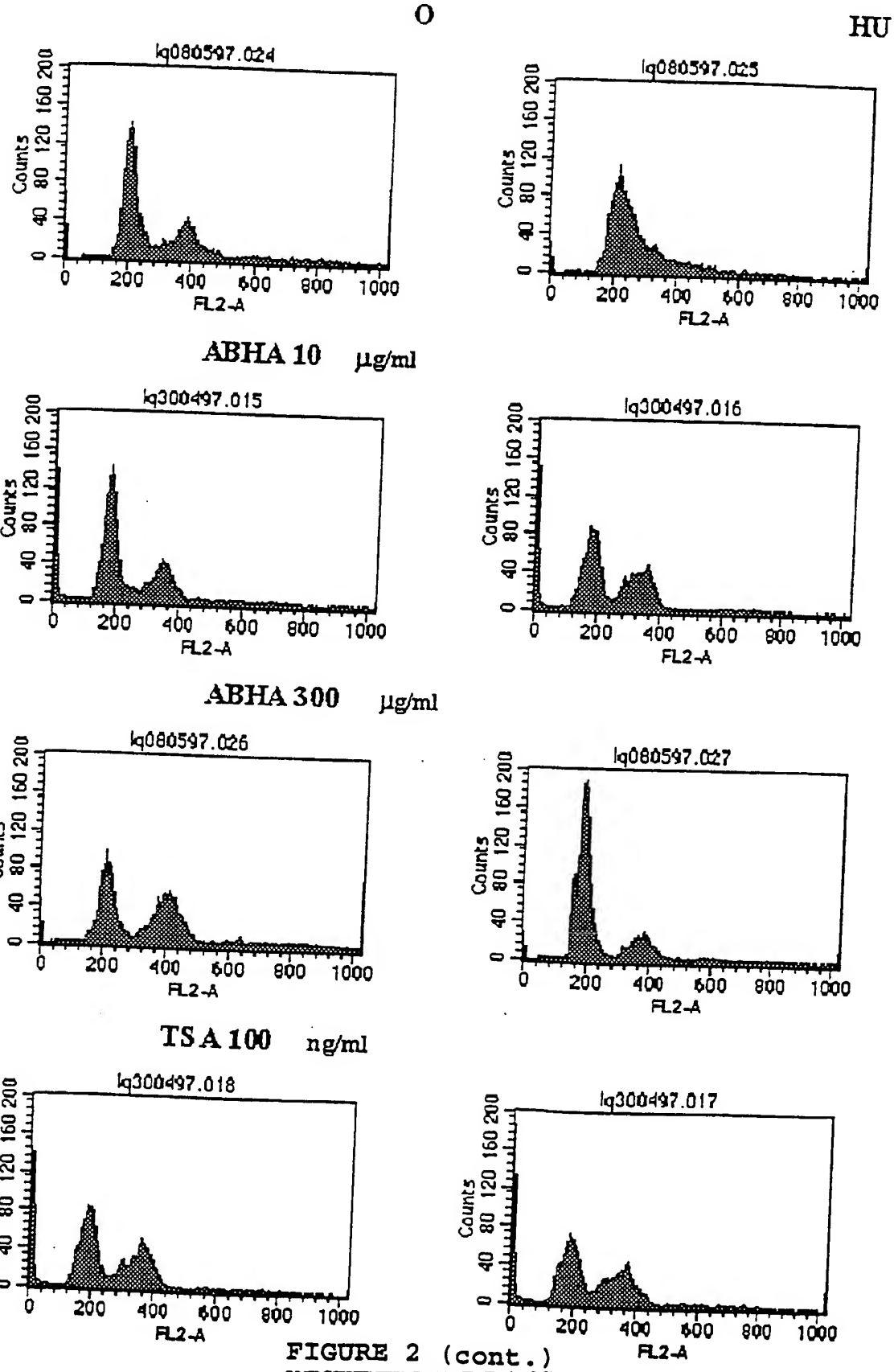


FIGURE 2 (cont.)

MM229 Resistant cells blocked in G2/M

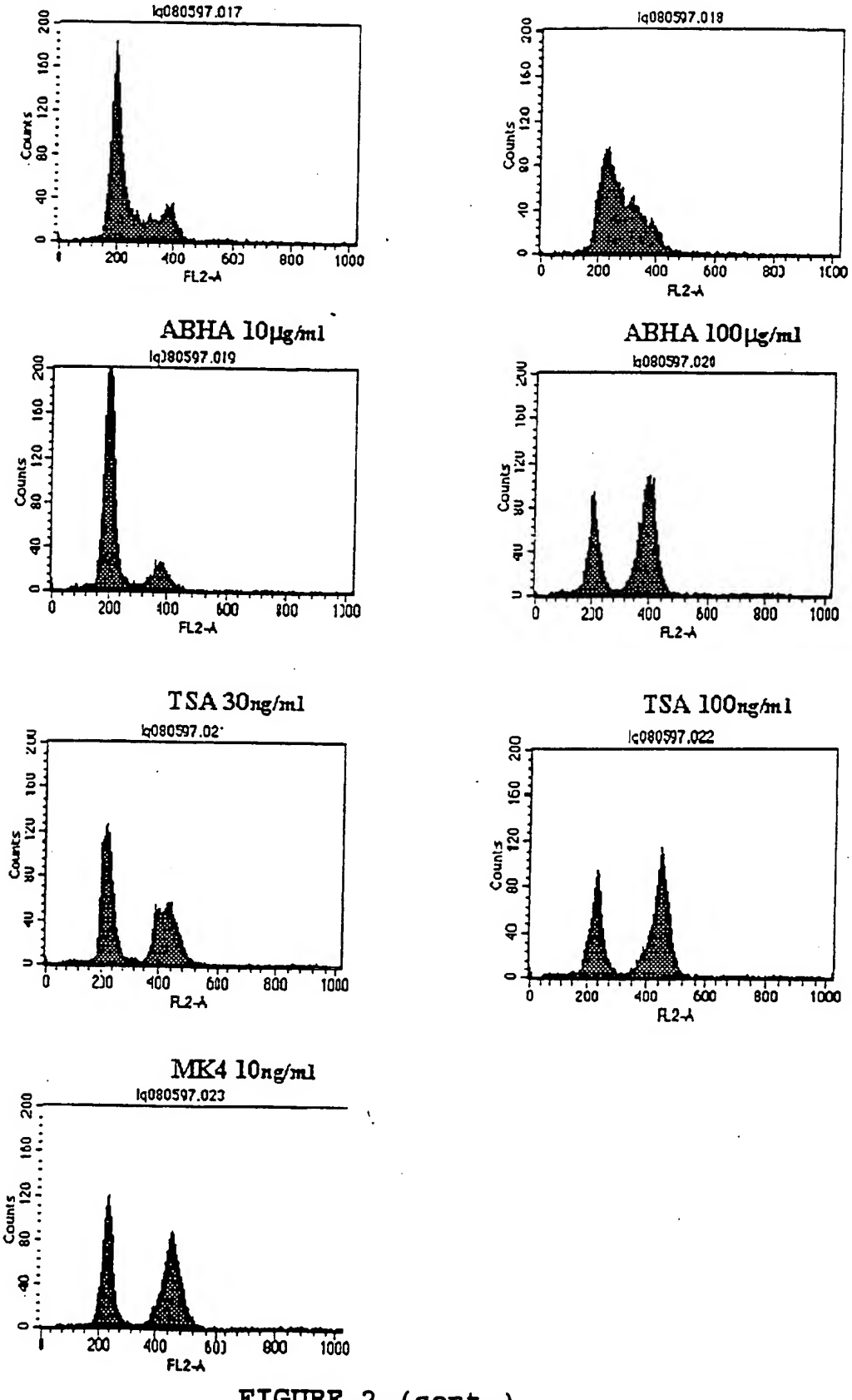


FIGURE 2 (cont.)

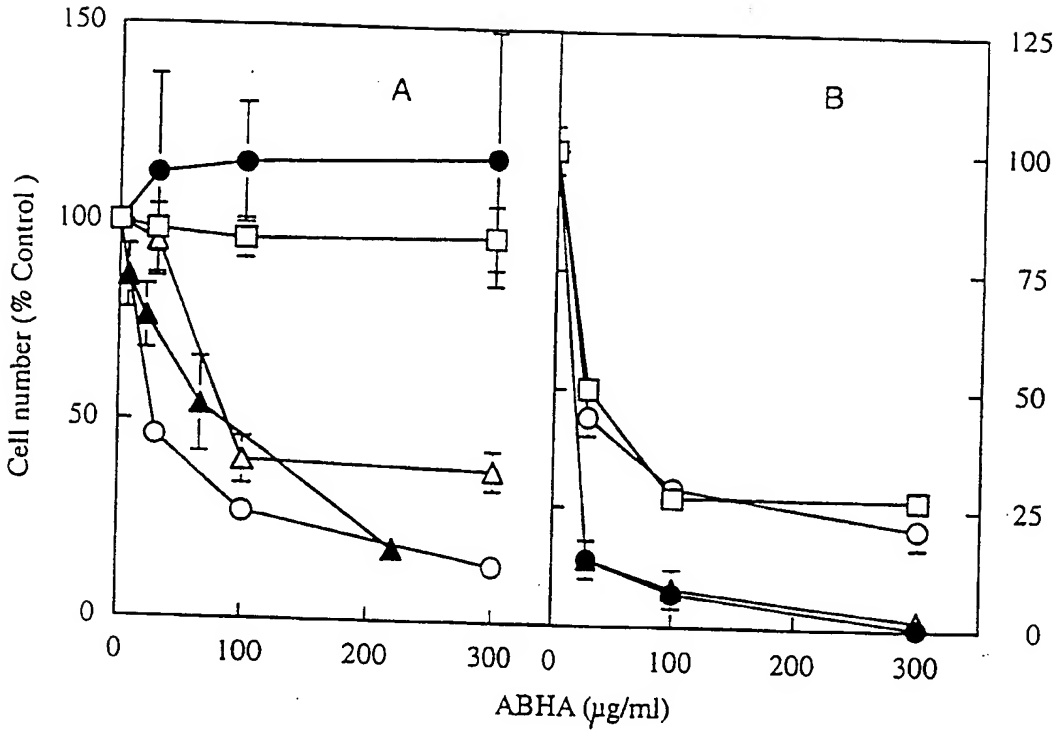

150
100
50
0
Cell number (% Control)
A
B
125
100
75
50
25
0
0
100
200
300
0
100
200
300
ABHA (µg/ml)

FIGURE 3

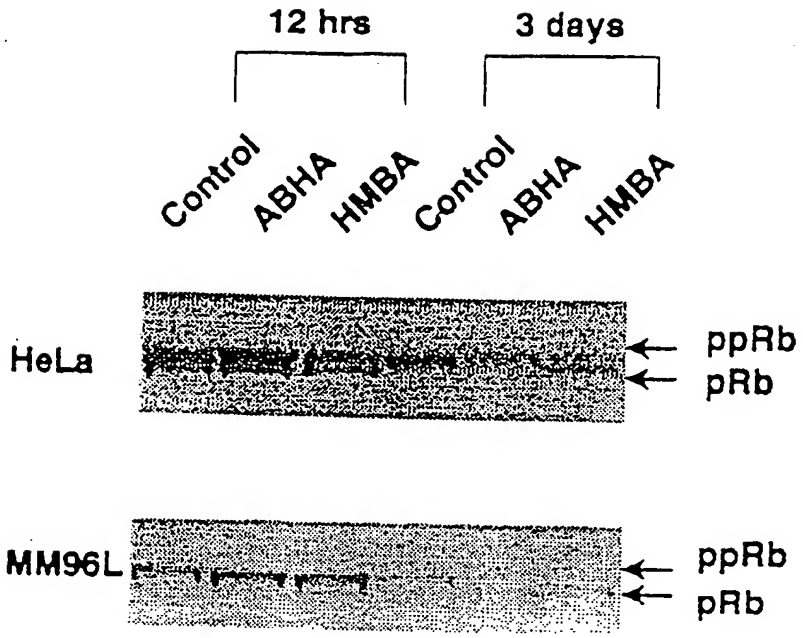

12 hrs
3 days
Control
ABHA
HMBA
Control
ABHA
HMBA
HeLa
ppRb
pRb
MM96L
ppRb
pRb

FIGURE 4

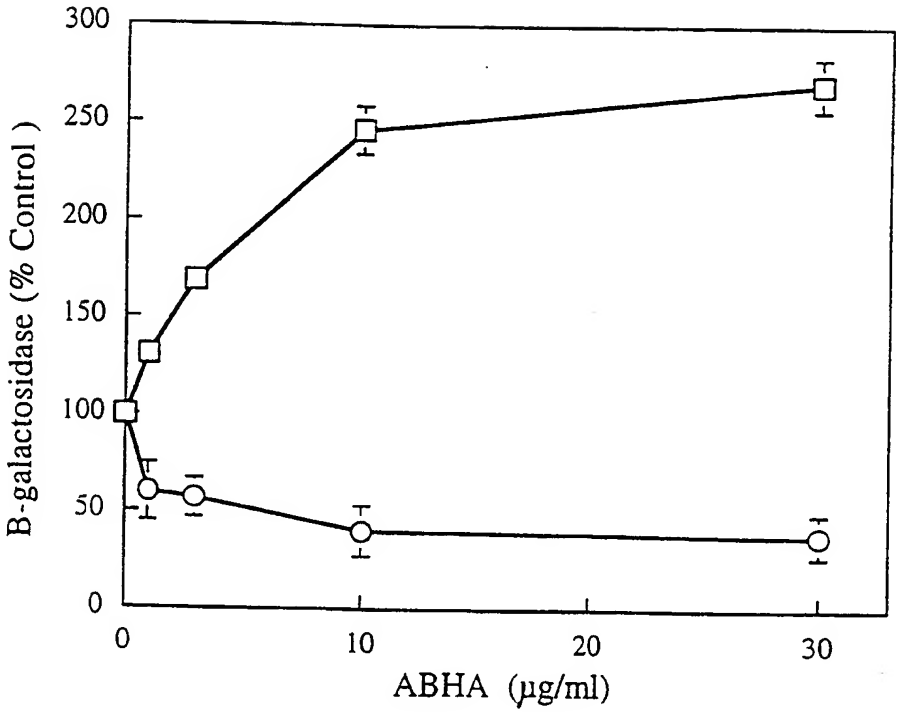

300
250
200
150
100
50
0
B-galactosidase (% Control)
0
10
20
30
ABHA (µg/ml)

FIGURE 5A

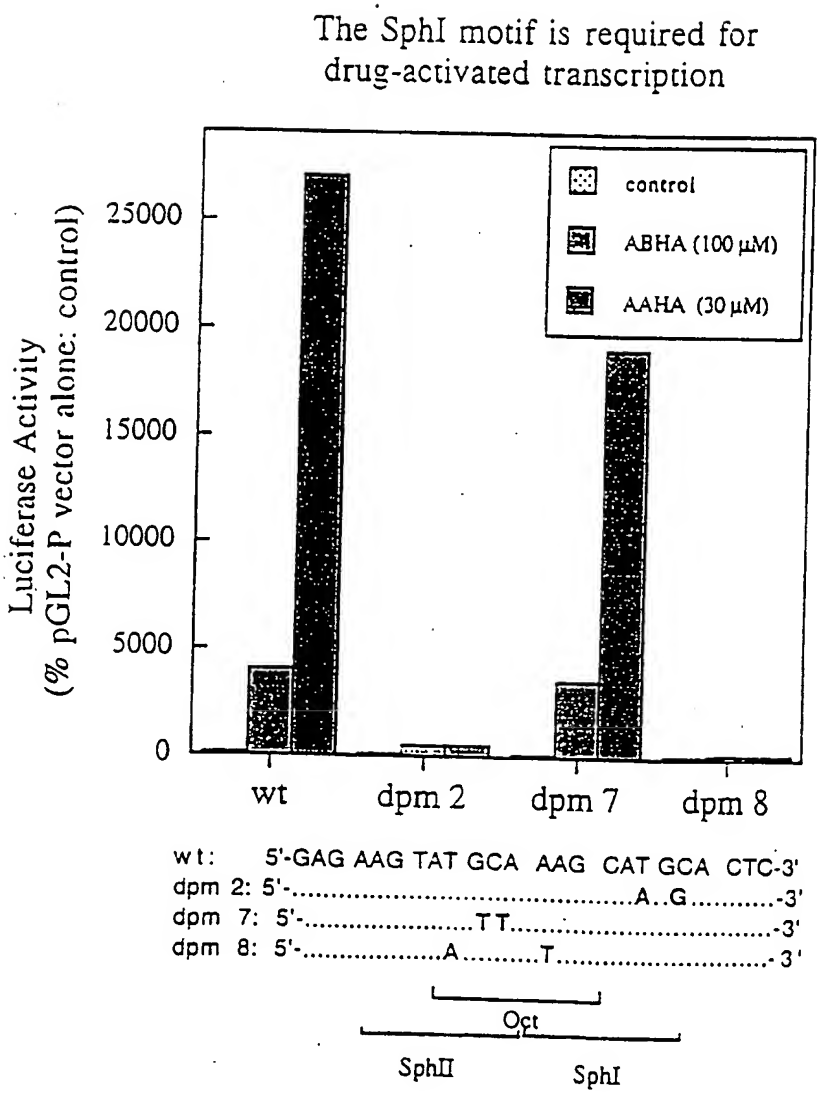

The SphI motif is required for drug-activated transcription
Luciferase Activity (% pGL2-P vector alone: control)
25000
20000
15000
10000
5000
0
control
ABHA (100 µM)
AAHA (30 µM)
wt
dpm 2
dpm 7
dpm 8
wt: 5'-GAG AAG TAT GCA AAG CAT GCA CTC-3'
dpm 2: 5'-.....A..G.....-3'
dpm 7: 5'-.....TT.....-3'
dpm 8: 5'-.....A.....T.....-3'
Oct
SphII
SphI

FIGURE 5B

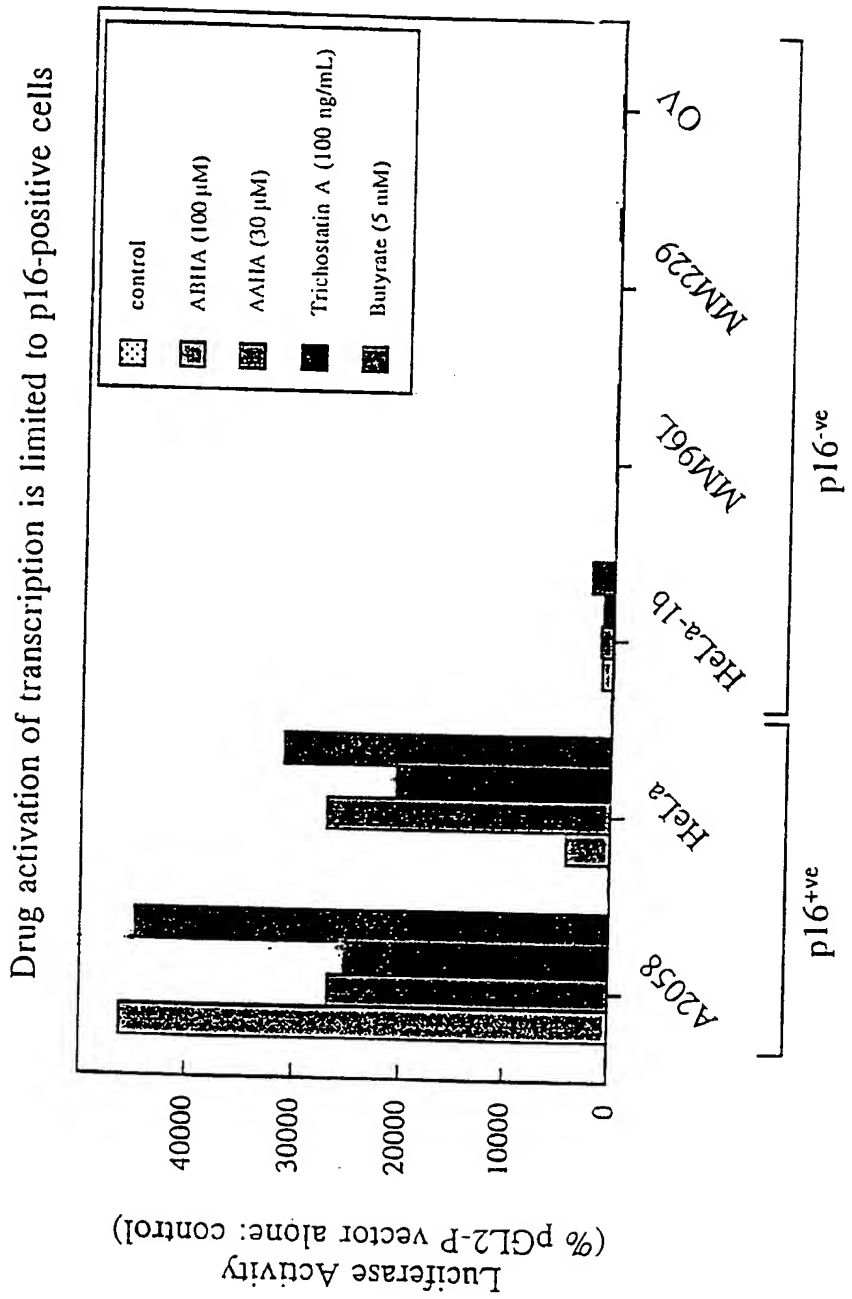
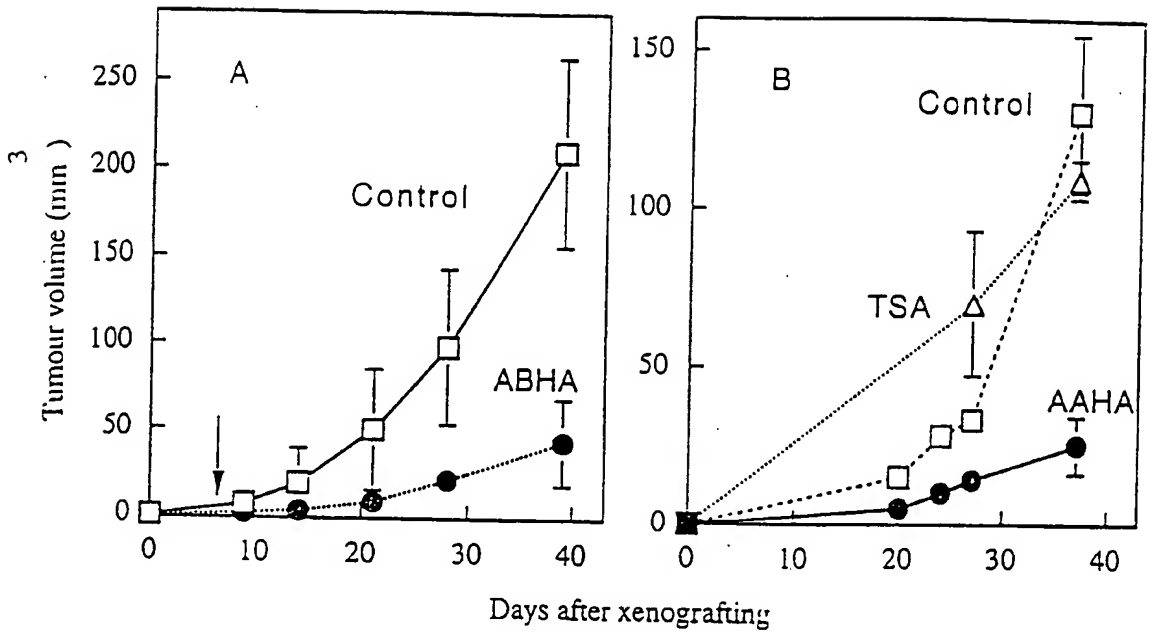

Drug activation of transcription is limited to p16-positive cells
Luciferase Activity
(% pGL2-P vector alone: control)
0
10000
20000
30000
40000
control
ABHA (100 μM)
AAHA (30 μM)
Trichostatin A (100 ng/mL)
Butyrate (5 mM)
A2058
HeLa
HeLa-1b
MM96L
MM229
OV
p16+ve
p16-ve

FIGURE 5C


A
B
Control
ABHA
TSA
AAHA
Tumour volume (mm 3)
Days after xenografting

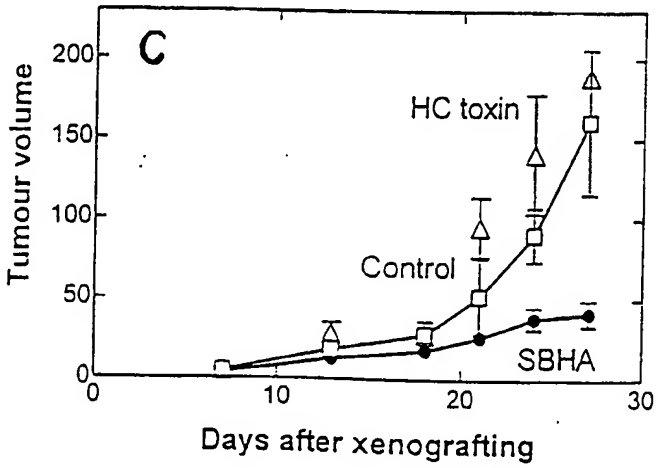

C
HC toxin
Control
SBHA
Tumour volume
Days after xenografting

FIGURE 6

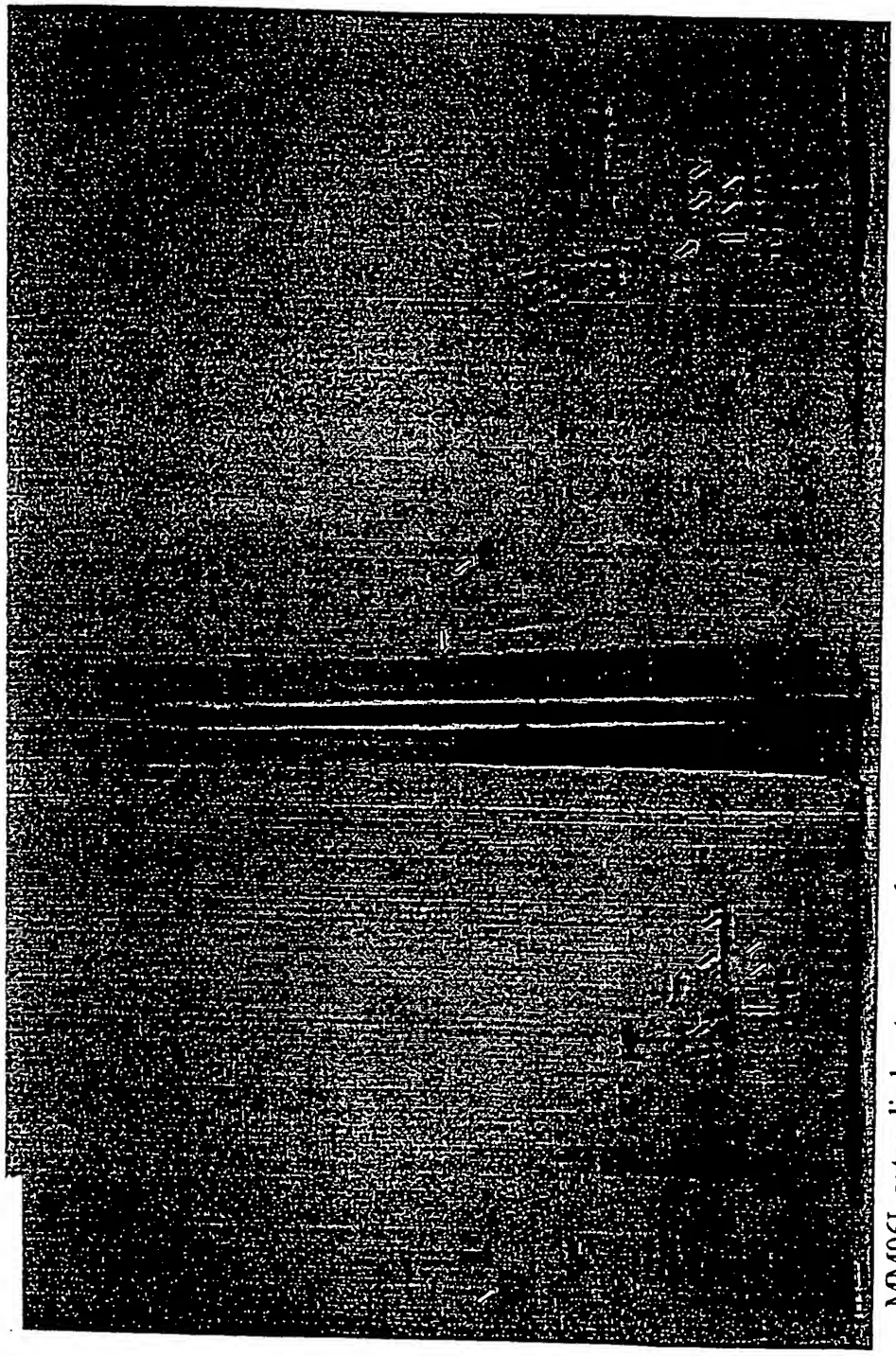

MM96L cytosolic lysate untreated control
cytosolic lysate from MM96 cell treated with 100μg/ml ABHA

FIGURE 7A

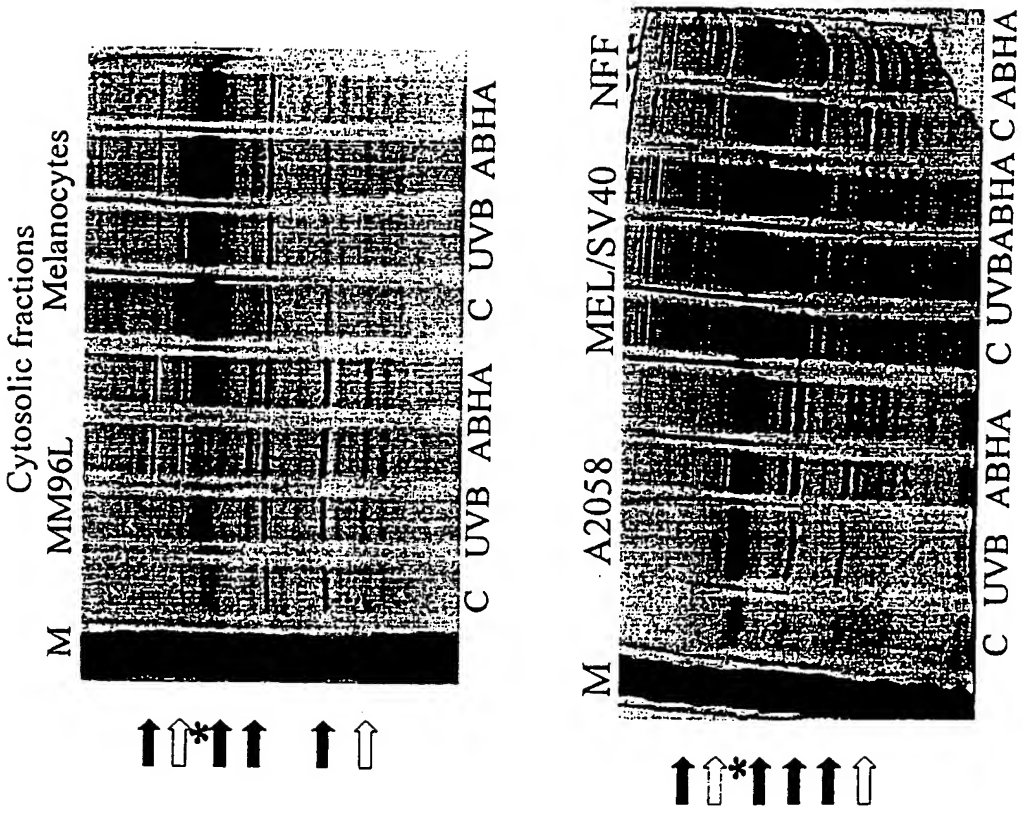

Cytosolic fractions
Melanocytes
M MM96L C UVB ABHA C UVB ABHA
M A2058 MEL/SV40 NFF
C UVB ABHA C UVBABHA C ABHA

FIGURE 7B

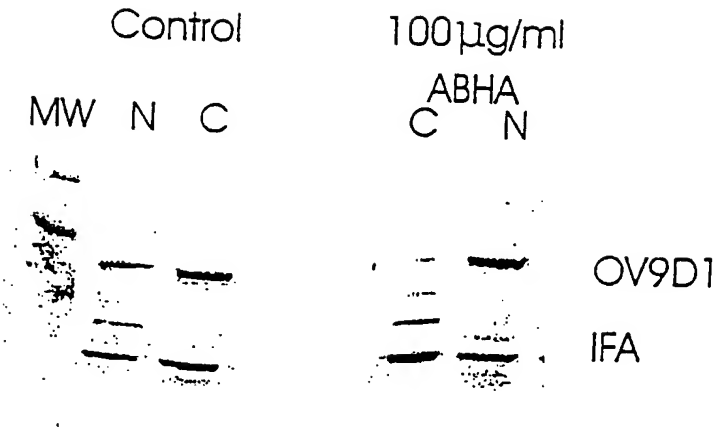


FIGURE 7C

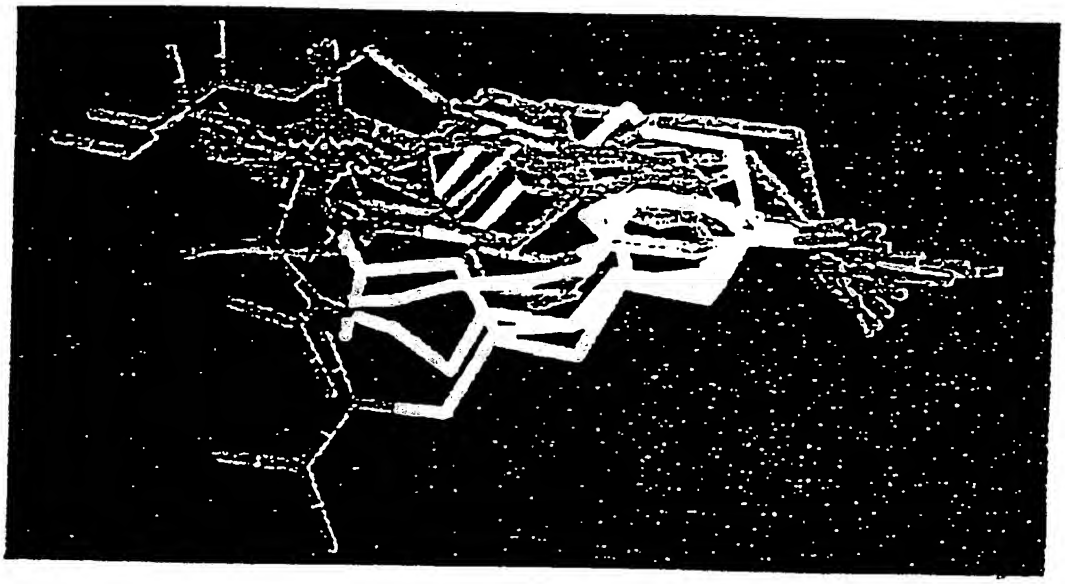


FIGURE 8

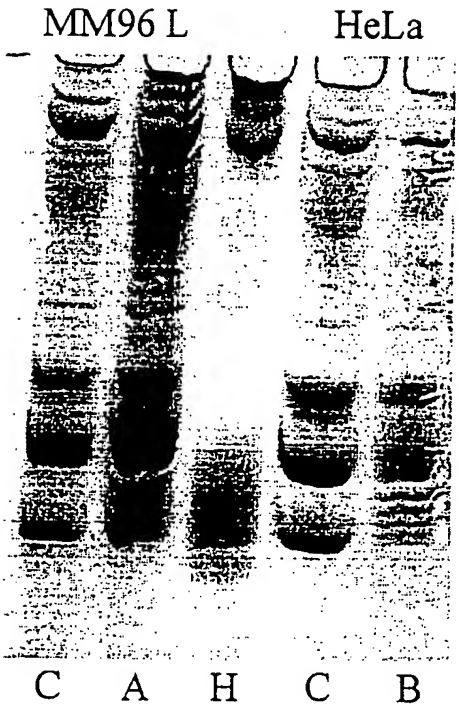


FIGURE 9A

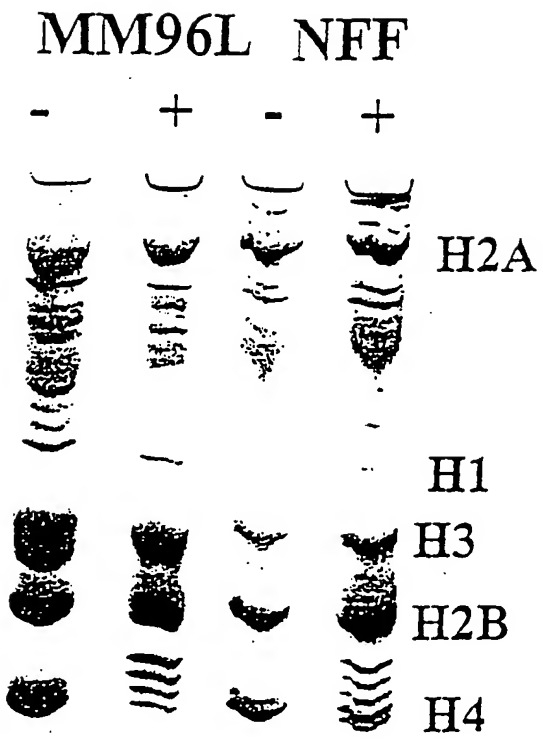


FIGURE 9B

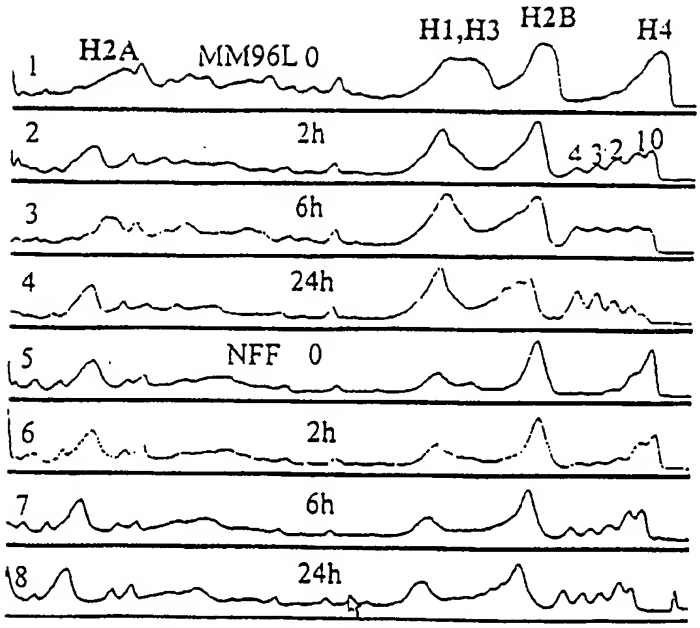


FIGURE 9C

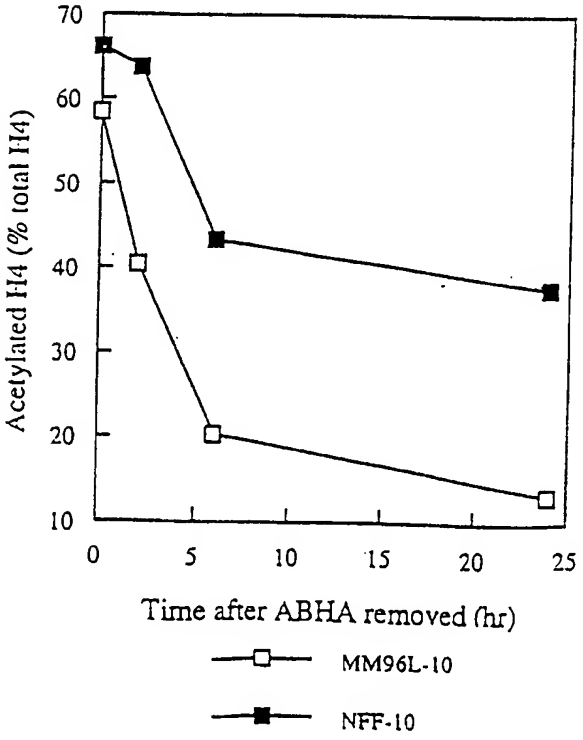
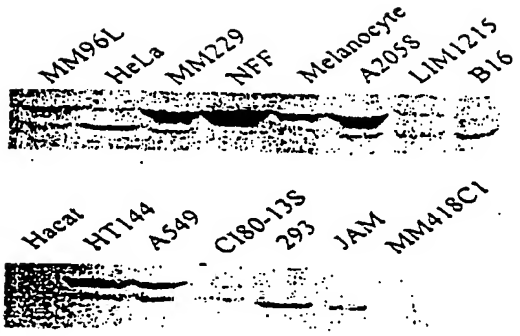


FIGURE 9D

A


MM96L
HeLa
MM229
NFF
Melanocyte
A2058
LIM1215
B16
Hacat
HT144
A549
CI80-13S
293
JAM
MM418C1

B

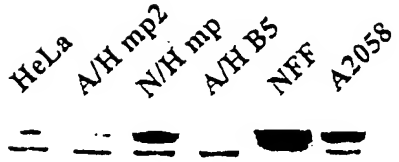
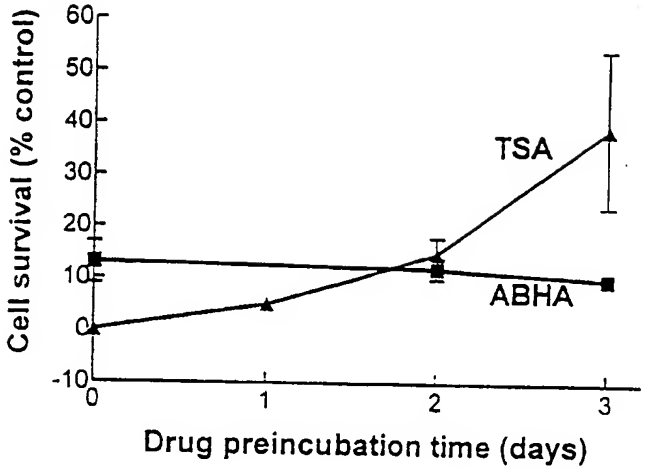

HeLa
A/H mp2
N/H mp
A/H B5
NFF
A2058

FIGURE 10

A


Cell survival (% control)
60
50
40
30
20
10
0
-10
0
1
2
3
TSA
ABHA
Drug preincubation time (days)

B

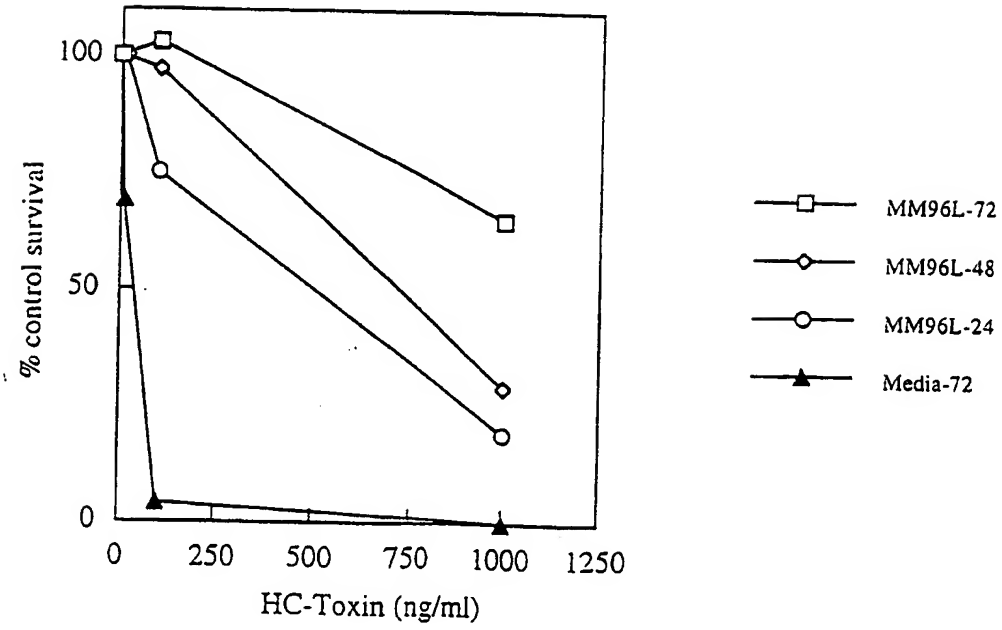

% control survival
100
50
0
0
250
500
750
1000
1250
HC-Toxin (ng/ml)
MM96L-72
MM96L-48
MM96L-24
Media-72

FIGURE 11

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00431

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07C 259/04, 259/06, 259/08, 259/10; C07K 5/04; C07D 209/44, 209/48, 217/22, 217/24, 217/14, 217/16; A61K 38/06, 38/05, 38/12, 31/16, 31/165, 31/38.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN: CHEM ABS. KEYWORDS: HYDROXAMIC (W) ACID. CANCER OR TUMOUR OR PARASIT? ALSO MOLECULAR FORMULA SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/05635 (CHIROSCIENCE LIMITED) 12 February 1998 (see whole document, see in particular pages 2 and 71)	1,2,7-9
X	WO 98/07697 (PFIZER INC) 26 February 1998 (see whole document in particular page 1)	1,2,8,9
X	WO 97/43249 (SMITHKLINE BEECHAM PLC) 20 November 1997 (see in particular the examples)	10,25
X	WO 97/24117 (RHONE-POULENC RORER PHARMACEUTICALS INC) 10 July 1997 (see whole document)	1,2,7-9

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 1998	30 JUL 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer **K LEVER** Telephone No.: (02) 6283 2254

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00431

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/42168 (ZENECA LIMITED) 13 November 1997 (see compound examples and general compound disclosure)	10.25
X	WO 97/43250 (SMITHKLINE BEECHAM PLC) 20 November 1997 (see compound disclosure pages 3,4)	10.25
X	WO 93/21942 (BRITISH BIO-TECHNOLOGY LIMITED) 11 November 1993 (see whole document)	1.2.7-11.23.24.32.43.44
X	WO 95/19961 (BRITISH BIO-TECHNOLOGY LIMITED) 27 July 1995 (see whole document)	1.2.5.7-11.23-26
X	WO 96/20918 (THE PROCTER & GAMBLE COMPANY) 11 July 1996 (see in particular pages 2-3)	1.2.5.7.8.9.14.22-26
X	WO 96/40101 (CIBA-GEIGY AG) 19 December 1996 (see pages 19-20, 27-28)	1.2.7-9
X	WO 94/10990 (BRITISH TECHNOLOGY LIMITED) 26 May 1994 (see whole document)	1.2.10
X	WO 93/07148 (SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH) 15 April 1993 (see whole document)	1.2.7-10.23-26
X	WO 95/31977 (SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH) 30 November 1995 (see whole document)	1.2.4-10.23-26
X	US 4 690 918 (BEPPU et al.) 1 September 1987 (see whole document)	1.2.4-10.23-26.38
X	JP ABSTRACT NO. 59-46244 (NISSAN KAGAKU KOGYO K.K) 15 March 1984	10.27-30.39-42
X	JP ABSTRACT NO. 07196598 (KURARAY CO LTD) 1 August 1995	10.43
X	WO 97/15553 (SANKYO COMPANY, LIMITED) 1 May 1997 (see abstract)	1.2.10.23-26
X	US 4 448 730 (BARTHOLOMEUS van't RIET et al.) 15 May 1984 (see in particular, column 1, lines 15-19; column 9 lines 35-45)	1.2.7.8
X	WO 93/12075 (SHIONOGI & CO LTD) 24 June 1993 (see abstract; also Derwent abstract 93-214037/26)	10.22
X	WO 97/31892 (SANKYO COMPANY, LIMITED) 4 September 1997 see abstract	10

INTERNATIONAL SEARCH REPORT

AUSTRALIAN PATENT OFFICE
SEARCH REPORT

Application No.
AU 98/00431

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document. with indication. where appropriate. of the relevant passages	Relevant to claim No.
X	International Congress of Chemotherapy. 6th. Tokyo 1969. Progress in antimicrobial and anticancer chemotherapy: proceedings 1970 "Antitumor activity of L-beta-aspartohydroxamic acid in vivo" Miura et al.. pages 170-174	1.2.7.8.9
X	The Prostate. Volume 34(2). 1998. "Cyclic Hydroxamic Acid Inhibitors of Prostate Cancer Cell Growth: Selectivity and Structure Activity Relationships". Roberts et al.. pages 92-99	1.2.7-9
X	Neoplasma. Volume 44(3). 1997. "Chloroaceto hydroxamic acid as an antitumour agent against Ehrlich ascites". Sur et al.. pages 197-201	1.2
X	Indian Journal of Pharmacology. 1997. Volume 29. "Antineoplastic activity of copper-benzo-hydroxamic acid complex against Ehrlich Ascites Carcinoma (EAC) in mice". Khanam et al.. pages 157-161	1.2
X	Anticancer Drugs. Volume 3. 1992. "The antineoplastic and cytotoxicity of benzohydroxamic acids and related derivatives in murine and human tumour cells". Hall et al.. pages 273-280	1.2.7-9
X	J. Med. Chem.. 1995. Volume 38. "Isolation and Characterization of a Cyclic Hydroxamic Acid from a Pollen Extract. which Inhibits Cancerous Cell Growth in Vitro". Zhang et al.. pages 735-738	1.2.7-9
X	Acta cientifica venezolana. Volume 32(5).1981. "Antitrypanosomal and antimycotic effect of various Hydroxamic Acids". Tabernero et al.. pages 411-416	10.27.28.39.40.42
X	Biochemical Pharmacology. Volume 53. 1997. "Tumor Selectivity and Transcriptional Activation by Azelaic Bishydroxamic Acid in Human Melanocytic Cells". Parsons et al.. pages 1719-1724	1.2.7.8.9
X	Journal of Pharmaceutical Sciences. Volume 84. No: 4. April 1995 "An in vivo model for Screening Peptidomimetic Inhibitors of Gelatinase A". Chander et al.. pages 404-409	10
X	Proc. Natl. Acad. Sci.. Volume 93. June 1996. "Second generation hybrid polar compounds are potent inducers of transformed cell differentiation". Richon et al. pages 5705-5708	1.2.10
X	Journal of Medical Chemistry. Volume 41(8). 1998. "Inhibition of Membrane-Type 1 Matrix Metallo proteinase by Hydroxamate Inhibitors: An examination of the Subsite pocket". Yamamoto et al.. pages 1209-1217 (see table 1)	1.2.10

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00431

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 21, 33-37 in full and 1-9, 11-20, 22-26, 31, 32, 38-44 in part
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claims are broad and speculative; much of what is claimed is not supported by the description (in particular claims 33, 34, 35, 36, 37). The claims are poorly drafted and as a result are unclear. Consequently it is impossible to determine the exact scope of the claims, for example.

Continued on extra sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Box I (continued)

(i) the meaning of the phrase "a compound structurally related thereto" is not clear (claim 1);

(ii) the term "acylaic" is not clear (claim 9);

(iii) some compounds of claim 15 do not fall within the scope of claim 10 to which they are appended;

(iv) the meaning of claim 21 is not clear, the compounds listed do not fall within the scope of claim 10 to which it is appended. The compounds are also well known;

(v) claim 27 is not clear because of the phrase "related compound as defined herein";

(vi) substituents are not defined within claims (claims 19 and 20);

(vii) compounds of claim 22 do not fall within the scope of claim 10 to which they are appended;

(viii) incorrectly appended claims (see claims 17, 23, 31 and 32);

(ix) the patent publication referred to in claim 10 is not related to hydroxamic acids or the current application.

These are some of the reasons that no meaningful search could be carried out on the above claims.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 98/00431

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	98/05635	AU	38564/97				
WO	98/07697	AU	34563/97				
WO	97/43249	AU	28973/97				
WO	97/24117	AU	15298/97				
WO	97/42168	AU	26454/97				
WO	97/43250	NONE					
WO	93/21942	AU	42672/93	EP	639982	ZA	93/03089
WO	95/19961	AU	14603/95	AU	16540/97	CA	2181709
		EP	740655	FI	962905	GB	2315750
		HU	74511	NO	963031		
WO	96/20918	AU	44220/96	BR	9510175	CA	2208679
		EP	800510	NO	973035	US	5639746
WO	96/40101	AU	61249/96	US	5646167	AU	52655/93
		CA	2112779	EP	606046	FI	940012
		HU	70536	JP	6256293	MX	9400276
		NO	940038	NZ	250517	SG	42933
		US	5455258	ZA	9400048	US	5506242
		US	5552419	US	5672615	AU	25369/95
		CA	2192092	EP	766672	FI	965156
		NO	965568				

Continued

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 98/00431

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	94/10990	AU	54301/94	EP	667770	US	5691382
WO	93/07148	AU	28703/92	AU	62063/96	EP	642509
		FI	941537	HU	67421	NO	941166
		US	5369108	US	5700811	AU	26474/95
		CA	2190765	EP	760657	WO	95/31977
WO	95/31977	NONE					
JP	07-196598	NONE					
WO	97/15553	NONE					
US	4448730	US	4263322	US	4394389		
WO	93/12075	EP	570594	US	5534654		
WO	97/31892	AU	18121/97	JP	9291072		

END OF ANNEX